









# NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

ANNUAL REPORTS *of the Director*

DIVISION OF INTRAMURAL  
RESEARCH

October 1, 1988 to September 30, 1989

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## PREFACE

Since its establishment as a separate Institute at the National Institutes of Health, almost four decades ago, the National Institutes of Diabetes and Digestive and Kidney Diseases (NIDDK) has conducted and supported research on many of the most serious diseases affecting the public health. Several of these are among the leading causes of disability and death in the nation; all affect seriously the quality of life of those afflicted with them. They constitute a tremendous drain, both direct and indirect, on the human and economic resources of the United States.

Our research programs encompass the various disciplines of internal medicine, less the cardiovascular system, allergy, and infectious diseases. Certain common scientific and biomedical denominators can be found in this broad array of diseases. Many of them overlap, with a common thread of molecular and cellular biology, endocrinology, metabolism, aberrant immune reactions, and nutrition running throughout. The outlook for advances in the treatment and prevention of these diseases is dependent on basic and clinical investigations into the nature of their interference with the normal functioning of the organ systems of the body.

A focus on basic research has traditionally guided the Institute's programs. It is grounded in the belief that a fundamental understanding of biological systems will ultimately elucidate the abnormalities underlying each disease and thus is imperative for the development of the most effective strategies for prevention and therapy. In addition to basic research, the Institute also has a commitment to expand advances in the understanding of disease processes into appropriate clinical studies and ultimately into efforts to transmit knowledge and effective technologies to practicing physicians.

This report chronicles the activities and advances of our intramural research program during the past year and indicates opportunities and plans for the future. Our intramural research staff has traditionally been acknowledged to be an innovative and highly productive group of scientists. The unusual caliber of this staff is reflected in a number of Nobel prizes and other prestigious awards that have resulted from its work. Concomitantly, scientists who trained in our intramural research laboratories and branches are among the leaders of the academic community throughout the country--fostering productive collaboration of our scientists with groups beyond the Bethesda campus.

NIDDK is justly proud of the contributions of its intramural research in FY 1989. Based on the gratifying



expansion of biomedical knowledge which we witness here and elsewhere in recent years, we wish to share with the readers of this report a feeling of encouragement and expectation for the years to come.

Phillip Gorden, M.D.  
Director  
National Institute of  
Diabetes and Digestive and Kidney Diseases



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Z01 DK 57504-02 LMCB  
Z01 DK 58000-44 LAC  
Z01 DK 58001-16 LAC  
Z01 DK 58002-14 LAC  
Z01 DK 58003-16 LAC  
Z01 DK 58004-22 LAC  
Z01 DK 58005-16 LAC  
Z01 DK 58006-06 LAC  
Z01 DK 58007-05 LAC  
Z01 DK 58010-04 LAC  
Z01 DK 58011-13 LAC  
Z01 DK 58012-03 LAC  
Z01 DK 58013-03 LAC  
Z01 DK 58014-02 LAC  
Z01 DK 58016-01 LAC  
Z01 DK 58501-03 LNS  
Z01 DK 59000-02 MPB  
Z01 DK 59001-24 MPB  
Z01 DK 59002-24 MPB  
Z01 DK 59501-03 LMC  
Z01 DK 59502-03 LMC  
Z01 DK 69000-24 PECR  
Z01 DK 69001-20 PECR  
Z01 DK 69006-19 PECR  
Z01 DK 69009-24 PECR  
Z01 DK 69015-07 PECR  
Z01 DK 69020-06 PECR  
Z01 DK 69021-09 PECR  
Z01 DK 69024-03 PECR  
Z01 DK 69025-03 PECR  
Z01 DK 69026-03 PECR  
Z01 DK 69027-02 PECR  
Z01 DK 69028-01 PECR  
Z01 DK 69029-01 PECR  
Z01 DK 69030-01 PECR  
Z01 DK 69031-01 PECR  
Z01 DK 69032-01 PECR  
Z01 DK 69033-01 PECR  
Z01 DK 69034-01 PECR  
Z01 DK 69035-01 PECR

#### INACTIVE PROJECTS

Z01 DK 19401-24 LC  
Z01 DK 19609-05 LC  
Z01 DK 29012-19 LCP  
Z01 DK 29015-18 LCP  
Z01 DK 29024-02 LCP  
Z01 DK 43214-05 MD

Z01 DK 45014-18 CEB  
Z01 DK 45020-13 CEB  
Z01 DK 47021-11 DB  
Z01 DK 54024-11 DDB  
Z01 DK 55011-07 MCNE  
Z01 DK 58015-02 LAC  
Z01 DK 69003-16 PECR

TRANSFERRED PROJECTS

FROM	TO
Z01 DK 58502-02 LN	Z01 DK 59501-03 LMC
Z01 DK 58503-02 LN	Z01 DK 59502-03 LMC

TERMINATED PROJECTS

Z01 DK 13019-05 MRB  
Z01 DK 19003-02 LC  
Z01 DK 25049-05 LCB  
Z01 DK 25067-02 LCB  
Z01 DK 45037-04 CEB  
Z01 DK 69014-12 PECR  
Z01 DK 69018-05 PECR



## ANNUAL REPORT OF THE MATHEMATICAL RESEARCH BRANCH

### National Institute of Diabetes and Digestive and Kidney Diseases

Current research projects of the Mathematical Research Branch reflect a broad range of interests in the development and application of theoretical models and of quantitative methodologies for understanding biological systems.

This research involves several different collaborations within the Branch and with other research groups, both at the NIH and elsewhere. This report describes recent work in the areas of molecular sequence analysis, electrical oscillations in nerve and secretory cells, synaptic neurobiology, microcirculation and facilitated transport, renal physiology, and selected other topics.

During the past year, international collaborative projects have involved foreign scientists at Hebrew University, Jerusalem (Department of Neurobiology), at The Weizmann Institute of Science, Rehovot, Israel (Department of Chemical Physics), and at the Universite Nationale Autonome de Mexico, Mexico City (Department of Mathematics); MRB scientists have been active participants in securing binational NSF grants for two of these projects. An invited presentation was given by J. Rinzel at the symposium, Classics in Theoretical Biology, Oxford UK. Several MRB scientists were invited lecturers (and chapter authors for a text) in the newly developed intensive course on Computational Neuronal Modeling (Marine Biological Laboratory, Woods Hole). A one-day symposium honoring the research contributions and 65th birthday of W. Rall was held at the NIH in 1988. D. Lipman, who has led an MRB subgroup on computational molecular sequence analysis, has been appointed Associate Director of the Center for Biotechnology Information, National Library of Medicine. J. Rinzel has been appointed as Adjunct Professor of Mathematics at the University of Maryland, College Park.

### Molecular Sequence Analysis

Multiple alignment of sequences. We have now implemented (with J. Kecioglu, U Arizona) in software our generalization of the Carrillo-Lipman algorithm and our gap cost methodology based on pairwise projections. The code can align up to seven amino acid sequences. It computes an evolutionary tree based on the pairwise distances, calculates weights for sequence pairs (see below), and uses a bounded shortest path approach to find an optimal alignment within an N-dimensional lattice. (D. Lipman & S. Altschul)

Our method which uses information from an evolutionary tree to derive weights for the pairwise distances of an SP measure was described previously. We have generalized this method to find weights for pairs of sequences which can be used to construct biologically realistic alignments. We have developed computational tools to compute these weights

and have evaluated their usefulness in constructing robust multiple alignments. Computation of the pairwise weights leads to a simple equation in matrix algebra, which we solved (S Altschul) with linear time graph theoretic methods and implemented computationally. (D. Lipman, S. Altschul, & R. Carroll, Texas A&M U)

Assume one is given a finite alphabet each of whose letters has an associated probability and an associated score. If at least one of these scores is positive, and the expected score is negative, there exists a "target probability distribution" such that the score associated with each letter can be expressed as the log of the letter's target probability over its associated probability. Given an infinite sequence of independent identically distributed random variables taking values from the given alphabet with the associated probabilities, the composition of the run of consecutive letters with greatest total score approaches the target distribution. This fact has important implications for the choice of scoring matrices for protein sequence comparison. (D. Lipman, S. Altschul, & S. Karlin, Stanford University)

Given a preparation of DNA from a collection of cells, partial digestion with restriction enzymes followed by dilution can yield aliquots in which a given marker has a 50% chance of being present. Two markers are present or absent concordantly, however, to a degree dependent on their physical separation. A given marker may be detected using the polymerase chain reaction. These observations provide a new strategy for doing genetic analysis, based upon digestion rather than genetic recombination. (S. Altschul & J. Silver, NIAID)

We have developed a method to find locally conserved regions in a set of amino acid sequences. This technique is used in an interactive tool to build protein motifs, and runs efficiently on small personal computers as well as workstations. The method is robust and can handle sequences which vary greatly in length and their degree of similarity. (D. Lipman & W. Pearson, U. VA)

Members of a large family of cell cycle control genes in yeast and mammalian cells act at different points in the cell cycle and have diverse biochemical functions. For example, CDC 2 is a protein kinase with 62% homology between yeast and humans. Another functionally-related set of gene products include enzymes involved in DNA synthesis, such as thymidylate synthetase and DNA polymerase. *S. cerevisiae* cell division cycle genes CDC 13, CDC 16, CDC 20 and CDC 23 provide functions required early in mitosis, but these functions are uncharacterized biochemically. The molecular cloning and sequencing of CDC 23 revealed multiple internal repeats within the protein as well as links to five other genes required for mitosis in three fungal species. The repeated sequence domains of these genes are currently being studied by in vitro mutagenesis to elucidate their mechanism of action. (D. Lipman & P. Hieter, Johns Hopkins University)

Apolipoprotein D is a member of a superfamily of proteins that bind small hydrophobic ligands and have diverse physiologic functions. Apo D itself is a component of high density lipoprotein (HDL) particles

and appears to have an important role in HDL metabolism, but its in vivo ligand has not previously been established. One member of this superfamily, insecticyanin (an insect camouflage protein), has been crystallized and its three-dimensional structure determined. Apo D is the most closely-related sequence to insecticyanin in the entire superfamily, and Apo D has been modeled using insecticyanin atomic coordinates. This model clearly predicts cholesterol and/or its ester as a ligand for Apo D. (D. Lipman & M. Peitsch)

### Electrical Oscillations in Nerve and Secretory Cells

The role of coupling in bursting electrical activity of pancreatic B-cells. We had previously demonstrated that cells which individually were unable to burst because of large perturbations due to stochastic potassium channel events could burst when coupled tightly by infinite conductance gap junctions. We have extended this model to allow for looser coupling by finite conductance gap junctions. Indeed bursting is attained for large coupling conductance, but surprisingly bursting is more regular for a range of finite conductance than if coupling is infinite. Furthermore, for an optimal conductance, the amplitude of cytosolic calcium oscillations is larger than for infinite conductance; the optimal value is near the physiological estimate. Since, with the looser coupling, spikes within a burst are not synchronized, current flow between the cells may account for the enhancements. We have explored this further with a simplified deterministic model in which two cells, each separately capable of bursting are coupled electrically. For an intermediate range of values of coupling conductance the cells burst synchronously, but with their spikes out of phase, and the calcium amplitude is increased. We have shown that the solution with in-phase spiking still exists but is unstable. (J. Rinzel & A. Sherman)

The model described above has been used to consider whether enhanced coupling could explain the reduction of burst frequency observed in low  $\text{HCO}_3$  buffers. An alternative hypothesis, that  $\text{HCO}_3$  affects the size of the internal bound calcium pool, leads to a better fit with available. (J. Rinzel, A. Sherman & P. Carroll, LCBG, NIDDK)

We have incorporated recent voltage-clamp data on calcium inactivation of calcium channels into the deterministic model for a single B-cell. Although previous models for calcium inactivation have been based on accumulation of calcium in a sub-membrane "shell", we reject this as unlikely for cells with low channel density, such as the B-cell. Rather, we propose that elevated calcium in the domain under an open channel binds to that channel to inactivate it. By adding this feature to an existing B-cell model in which burst onset and termination are regulated by slowly accumulating cytosolic calcium which activates potassium channels, we show that calcium inactivation on the faster time scale of a single spike may play no role in bursting, counter to the suggestions of others. (A. Sherman, J. Rinzel & J. Keizer, Univ Calif-Davis)

We continue to develop the averaging method for models of bursting oscillations in excitable cells. In this technique, the details of fast

action potential dynamics are not computed instantaneously, only their average affect on the slow processes is included, e.g. the net change in intracellular calcium concentration per action potential. A reduced model for only the "averaged" slow processes is studied. Our previous idealized model of parabolic bursting (which includes action potential phase but not amplitude) was refined; it has one fast and two slow variables. With analytical and numerical methods we have identified parameter regimes in which the model exhibits resting or bursting or continuous spiking behaviors. We have verified empirically that the averaged equations approximate well the slow variable time courses of the full three-variable model. (J. Rinzel, S. Baer & H. Carillo-Calvet, Nat. Auto. Univ. of Mexico)

Anterior hypothalamic hormones are delivered to the pituitary in a periodic, pulsatile fashion; for GnRH the period is 1-2 hours. The period, and so the total amount of GnRH secreted is sensitive to estradiol secreted by the ovaries. This forms a feedback loop. A simple physiological model (not yet mathematical) of pulse generation is proposed. It involves hypothalamic secretory neurons impinging on inhibitory GABA neurons that are sensitive to steroids. Low level activity of the secretory neurons is proposed to cause a calcium flux into the GABA neurons. When an internal calcium ion threshold is reached the inhibitory neurons are shut off. The size of the protein calcium sink is steroid-moderated. Branching interconnection of both the secretory and inhibitory neurons could yield the observed concerted activity of the secretory neurons. (M. Weinstein & J. Rinzel)

#### Excitability and Impulse Propagation

Previously, we formulated and applied a kinematic description for the position-time trajectories of nerve impulses during propagation. Each impulse is treated as a discrete entity, but its instantaneous velocity is not generally constant because it is affected by the time course of predecessor recovery. Computations with this approximate model are much simpler than with the full nonlinear cable equations, and empirical comparisons show good agreement. We have now developed a formal derivation (previously lacking) for the kinematic equations in the limit of large interspike intervals. This derivation more explicitly connects the parameters of recovery to the kinematic model, and facilitates characterization of three generic types of recovery and conduction properties. (J Rinzel & E Meron, Weizmann Institute) A Hodgkin-Huxley-like model has been formulated to describe the low threshold spike (LTS) in thalamic neurons. Data from whole cell recordings (Rogawski, NINDS) are used to fit the model. The major contributor to the LTS is a voltage-activated and inactivated calcium (T-type) current; potassium currents may also be involved. With only the calcium current the model mimics the qualitative and quantitative behaviour seen experimentally (Rogawski). The introduction of a fast potassium (A-type) current thought also to be involved extinguishes the LTS in the model. It is unclear whether this is a failing of the model or whether this is indicative of the physiology. (J. Rinzel, M. Weinstein & M. Rogawski, NINDS)

## Synaptic Neurobiology

Previously, a computational network of 64 coupled Hodgkin-Huxley cable neuron models was implemented on the NCI's Cray X/MP, a vector processing Supercomputer. While the optimized vector code was much faster than in scalar mode, scaling considerations suggested that the computations might be better adapted to a massively parallel computer. The network solver, and several other software utilities, were therefore implemented on the Naval Research Laboratory's Connection Machine (CM-2). (M. Mascagni)

Clusters of excitable dendritic spines. Important implications for local processing of synaptic inputs to dendritic spines were presented and discussed in previous reports and publications. A new set of computations is being designed, using different values for several key parameters. Comparison of these results with our earlier results will provide the basis for a comprehensive accounting of the functional implications of excitable dendritic spine clusters. (I. Segev, Hebrew Univ. & W. Rall)

Implications of neural models for nerve network computations. There is growing interest in nerve network modeling; however, such models have so far assumed very simplified neuronal properties. We plan to explore network models which incorporate nonlinear properties and spatio-temporal aspects that we have previously explored in our neural modeling. Also, an invited seminar on this subject was presented to the Santa Fe Institute (May 1989). This Institute sponsors workshops and collaborative networks in several different research areas involving complex systems (in physics, biology, computer science, and economics). Agreement was reached to plan a future workshop to encourage cooperation between neural modelers and network modelers; this workshop would explore the problems and the rich possibilities to be expected when reasonably realistic neural models are incorporated into nerve network models. (W. Rall)

Estimating the electrotonic structure of neurons. Work has continued on finding efficient ways to estimate the electrotonic structure of neurons whose dendritic trees cannot be approximated as equivalent cylinders. In particular we have explored how a "reduced" model might be used to get preliminary estimates of the electrotonic parameters which can then be used to solve the "inverse" model using all of the known morphological information. We are trying to find conditions in which a reduced model might be appropriately used. (W. Holmes & W. Rall)

Long-term potentiation (LTP). The induction of long-term potentiation is thought to depend on  $\text{Ca}^{2+}$  influx through NMDA receptor channels. We continued to study  $\text{Ca}^{2+}$  influx through NMDA receptor channels using a revised, detailed model of a hippocampal dentate granule cell. Both our old model and our revised model found the same steep transition from low to high  $\text{Ca}^{2+}$  influx with increasing input frequency given a sufficient number of coactivated inputs. Since the difference between low and high  $\text{Ca}^{2+}$  influx in both models was at most four-fold, we mod-

eled free  $\text{Ca}^{2+}$  concentration in the spine head as a function of input frequency and the number of co-activated synapses. Because of the various  $\text{Ca}^{2+}$  reactions that occur in the spine head, the 2-4-fold differences in  $\text{Ca}^{2+}$  influx through NMDA receptor channels was magnified into 20-30-fold differences in spine head  $\text{Ca}^{2+}$  concentration. Although our previous model did not accurately predict the best temporal input patterns for associative LTP found experimentally, this revised model does. It also provided insights into the kinetics of the interactions between neurotransmitter and NMDA receptors which bring about the opening of NMDA receptor channels. (W. Holmes & W.B. Levy, Univ. VA)

#### Microcirculation and Facilitated Transport

The purpose of this work is to develop mathematical models of the blood flow and transport in the microcirculation. An effort is made to incorporate in the models the histological structure of the microcirculation, the blood and tissue transport parameters from available experimental observations. The diffusion and consumption of oxygen in the presence of myoglobin in the tissues is studied. A two dimensional model with discrete mitochondria in exercise is developed. The arteriolar tone and vasomotion is being studied as well as their relation to the control of the peripheral blood flow; physiological implications for the microcirculation in sickle cell anemia are being considered (with A Schechter & G Rodgers, LCB, NIDDK) and preliminary mathematical models are being formulated (with G.B. Ermentrout) (J.M. Gonzalez-Fernandez)

#### Renal Physiology

We have used the multi-nephron, central core model of whole kidney (see previous reports) to evaluate three experimentally observed effects of atrial natriuretic factor (ANF) on renal NaCl and water excretion. Simulations show that inhibition of collecting duct active NaCl absorption by 50% or more can increase NaCl and water excretion to levels that match experimental values; predictions that urinary sodium concentration will increase to greater than plasma levels agree with experiments. Decreased collecting duct water permeability predicts an increase in water excretion with little change in NaCl excretion, and simulated increases in glomerular filtration rate of 2.5-5% also increase NaCl and water excretion rates to observed levels in response to ANF. However, this action is less effective than inhibition of collecting duct active NaCl absorption in increasing urinary [NaCl]. We conclude that a combination of several actions are likely to account for the overall renal effect of ANF. (R. Mejia, M. Knepper, NHLBI, J. Sands, NHLBI, & J.L. Stephenson, Cornell U Med)

We continued development of a rigorous description of pH balance that is based on physical principles. Previously, we described a canonical tube model for solute, flow, and charge conservation including individual reactive species and chemical buffers, and we simulated perfusion experiments of isolated rabbit cortical collecting ducts. We have now extended this model to describe a tubule embedded in an interstitium. Simulated variables, including non-reactive ions, neutral species and electrical potential, are obtained as functions of a prescribed interstitial profile. We have used an initial-value-problem

version of this model to study numerically the transport properties of the thick ascending limb of Henle, and we are implementing a solution of the boundary value problem using CONKUB (described previously). (R. Mejia & M. Knepper, NHLBI)

#### Selected Other Topic Areas

Ovulation control. Some numerical experiments were performed which included random perturbations of steady state solutions to an integro-differential equation modeling ovulation control in mammals. These calculations yielded further evidence that the region of stability in function space of steady state solutions shrinks with decreasing follicular mean entry rates. This work complements previous analytical and numerical work with the continuous model which showed the existence, uniqueness, stability, and behaviour of steady state solutions for all mean activation rates. A manuscript has been submitted for publication. (M. Weinstein)

Cell energetics. As described previously, a diffusion-reaction model for ATP and its byproducts has been used to study concentration profiles in a renal cell during transitions in ion transport. We have now shown that a phosphorylation rate proportional to a product of [ADP] and [Pi] in our previous simulations is consistent with an apparent Michaelis constant for ADP of 100  $\mu$ M. As the pump rate increases to 10 times control, the model shows that a value of  $K_m, ADP = 56 \mu$ M, which has been observed experimentally, yields [ATP] at the mitochondria equal to the control value with a proportional increase in ATP delivery to the pump sites and no apparent diffusion limitation. (R. Mejia, R.M. Lynch, NHLBI & R.S. Balaban, NHLBI)

Numerical solution of boundary value problems (BVPs) for elliptic partial differential equations. A Wiener integral (probabilistic) representation for elliptic BVPs has been implemented on a massively parallel computer (CM-2, see above). With an adjoint random walk approach, walks emanating from the boundary of the region are utilized for a more efficient sampling of the solution. Analysis has shown that the expected value of an appropriate statistic can be represented as the quotient of solutions of two related parabolic initial-BVPs. This provides a deterministic iterative solution to the elliptic BVP which is empirically as efficient as the optimal stationary iterative method. (M. Mascagni)

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 13,001-16 MRB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mathematical formulations and analysis relevant to experimental neurophysiology.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: W. Rall Senior Research Physicist MRB, NIDDK

Others: W. R. Holmes Staff Fellow MRB, NIDDK

## COOPERATING UNITS (if any)

Dept. of Neuroscience, Hebrew Univ. of Jerusalem

## LAB/BRANCH

Mathematical Research Branch

## SECTION

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.8

## PROFESSIONAL:

2.5

## OTHER:

.3

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

RESEARCH AREA. Basic neuroscience involving structure/function relations for neuronal dendritic branching, dendritic spines, and synapses (also neuron populations with cortical symmetry), and for such functions as synaptic transmission, amplification and dendro-dendritic interactions in the context of spatio-temporal input patterns, logical processing of input, and neural plasticity, as in conditioning and learning.

RATIONALE. Combine experimental data from neuroanatomy and from electrophysiology with biophysical models of nerve membrane (passive, synaptic and excitable) into a comprehensive theory which can lead to new insights and to testable theoretical predictions (leading to the design of better experiments). To do this we must create explore and test mathematical and computational models with different degrees of complexity.

METHODOLOGY. Our methods include both analytical solutions and computational solutions of boundary value problems (for partial differential equations) in the tradition of classical physics. They include also the formulation and solution of problems in terms of systems of ordinary differential equations; when this is done explicitly for a compartmental model of a neuron, it is possible to accommodate a remarkable variety of dendritic branching patterns and non-uniform distributions of membrane properties and of synaptic inputs.

RESULTS. Earlier results are summarized in Chapt. 3 of "The Handbook of Physiology: The Nervous System, Vol. 1" published by the American Physiological Society, 1977 (Kandel, Brookhart & Mountcastle, eds.). More recent results are described in Chapters 22 and 24, in "Cellular Mechanisms of Conditioning and Behavioral Plasticity" (eds. Woody, CD, Alkon, DL & McGaugh, JL) Plenum Press, 1988.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 13,002-17 MRB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mathematical description of substrate transport in capillary-tissue structures.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. M. Gonzalez-Fernandez Research Mathematician MRB, NIDDK

## COOPERATING UNITS (if any)

Dept. of Mathematics  
Univ. of Pittsburgh

Lab. of Chemical Biology, NIDDK

## LAB/BRANCH

Mathematical Research Branch

## SECTION

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.53

## PROFESSIONAL:

1.5

## OTHER:

.03

## CHECK APPROPRIATE BOX(ES)



(a) Human subjects



(b) Human tissues



(c) Neither



(a1) Minors



(a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

The goal of this work is to develop mathematical models of the blood flow and transcapillary exchanges in capillary networks. An effort is being made to incorporate in the models of the histological structure of capillary networks as well as different flow patterns from available experimental information. In this model the extraction of substrates with different chemical kinetics at the tissue site will be described. It is expected that this could be used in experimental situations where the extraction of different substrates are measured simultaneously, thus helping to infer the flow pattern features of the microcirculation. In particular a model of the diffusion-consumption of oxygen in striated muscle containing myoglobin (facilitated diffusion) is being developed and pertinent numerical results examined.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 13,004-15 MRB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mathematical description of cellular neuroelectric signal transmission.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. Rinzel	Chief, MRB	MRB, NIDDK
Other:	A. S. Sherman	NRC Fellow	MRB, NIDDK
	M. V. Mascagni	NRC Fellow	MRB, NIDDK
	M. H. Weinstein	NRSA Fellow	MRB, NIDDK

COOPERATING UNITS (if any) Dept/Mathematics, U/Pittsburgh; Med. Neurology Br, NINCDS  
 Lab/Cell Biol & Genetics, NIDDK; Dept/Chem Physics, Weizmann Inst. ISRAEL  
 Dept/Chem, U/California, Davis; Dept/Mathematics, UNAM, Mexico City, MEXICO  
 Dept/Mathematics, Arizona St. Univ.

## LAB/BRANCH

Mathematical REsearch Branch

## SECTION

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

4.5

## PROFESSIONAL:

4.0

## OTHER:

.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project continues to focus on the formulation, analysis, and biophysical interpretation of mathematical models which describe various aspects of neuroelectric signaling for individual neurons. Among the topics of current interest are: (i) integration of synaptic input delivered to the soma and dendritic branches of a neuron; (ii) propagation of action potentials along axons; (iii) stimulus-response and threshold properties for repetitive-firing of action potentials; (iv) complex bursting patterns of membrane potential oscillations which arise through endogenous membrane properties and/or interneuronal coupling.

Because qualitatively related mathematical or biophysical problems may arise in other contexts, e.g. chemical and biochemical oscillations, or e.g. excitation-secretion coupling, this project may consider models from such applications.

Mathematical models of these phenomena involve systems of linear and nonlinear ordinary differential equations and parabolic partial differential equations. Solutions and their mathematical stability are determined by analytical and numerical methods drawn from both classical and modern applied mathematics. These methods may include finite difference or finite element numerical integration, bifurcation theory, perturbation techniques, and nonlinear dynamical systems theory. One goal of this project is to expose the qualitative mathematical structure for classes of models by exploiting simple, yet physiologically reasonable, equations.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 13,014-08 MRB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Probabilistic Analyses of Nucleic Acid Sequences.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. J. Lipman	Research Scientist	MRB, NIDDK
Other:	S. F. Altschul	IRTA Fellow	MRB, NIDDK
	M. S. Boguski	Medical Staff Fellow	MRB, NIDDK

COOPERATING UNITS (if any) Math/Dept, Stanford U; Statistics/Dept, Texas A&M U;  
Computer Sci/Dept, U/Arizona; NIH, NIAID; Lab/Math Biol, NCI; FCRF, NCI;  
Biochem/Dept, U/Virginia Sch/Medicine;  
Dept/Molecular Biol & Genetics, John Hopkins U/Sch/Medicine

## LAB/BRANCH

Mathematical Research Branch

## SECTION

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.54

## PROFESSIONAL:

2.5

## OTHER:

.04

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This project has focussed on the analysis of amino acid and DNA sequence data as it pertains to molecular biology and molecular evolution. Areas of interest have included: The description of measures of similarity appropriate for protein sequence comparison; the development and analysis of algorithms for multiple sequence alignment and their implementation as useful software; the formulation and solution of mathematical problems with relevance to biological sequence comparison; the analysis of biological sequence data for evolutionary and functional relationships.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 13,017-06 MRB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Sound processing in the auditory system.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	S. A. Shamma	Guest Worker	MRB, NIDDK
Others:	J. Rinzel	Chief, MRB	MRB, NIDDK
	R. Chadwick	Biomedical Engineer	BEI, DRS

## COOPERATING UNITS (if any)

Biomedical Engineering &amp; Instrumentation Br., DRS

## LAB/BRANCH

Mathematical Research Branch

## SECTION

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.4

## PROFESSIONAL:

1.2

## OTHER:

.2

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

The project involves research on the processing of speech and complex sounds in three areas:

1. Neural network models to process and extract important parameters of speech and other sounds for both monaural and binaural hearing at the early levels of the auditory system.
2. Models of the cortical processing and representation of complex sounds.
3. Learning algorithms mimicking adaptive central auditory neural networks to perform storage and recognition tasks.

## DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 13,019-05 MRB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mathematical study of excitability properties in coupled nerve membrane patches.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S. M. Baer

Staff Fellow

MRB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Mathematical Research Branch

## SECTION

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This project has been terminated.

## ANNUAL REPORT OF THE LABORATORY OF CELLULAR AND DEVELOPMENTAL BIOLOGY NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

A broad spectrum of types of research are sheltered under the umbrella of cellular and developmental biology as studied by the forty scientists and support personnel of LCDB. At one end of this spectrum are atomic and molecular resolution studies of proteins and protein-DNA complexes; at the other are studies of mammalian development and genetic defects. Between these are groups interested in genetics and gene regulation in yeast, *D. discoideum*, sea urchins and human tissue culture cells; ultrastructure; biotechnology; hormonal regulation mechanisms and oncogenes. Most of the groups share common technological approaches in their work; this, together with an environment which fosters communication, leads to highly productive interactions among all groups within the laboratory, in spite of the seeming diversity of research areas under investigation.

The past year has seen significant progress in all the areas studied in LCDB. In addition to research papers, members of the laboratory have published several critical invited reviews and presented data at universities, other NIH laboratories, and national and international meetings. Of particular note this year was the first international meeting on ribonucleases in Moscow. Robert Hartley attended and presented a lecture. Fully 25% of the papers offered at that meeting studied barnase and/or barstar, the proteins Hartley has developed as a system for study of protein folding and protein-protein interactions.

The following summary of research in LCDB for 1988-1989 is organized as we have done previously. Rather than summarize experimental work done by individual Sections or working groups, I choose to review progress in a thematic sense, by the types of research done in the laboratory. This approach emphasizes the continuity of the spectrum of research done in LCDB and will hopefully lead the reader to an appreciation of the interactions which make the laboratory more than the sum of its component parts.

### STRUCTURAL STUDIES

The use of barnase, an extracellular ribonuclease of *B. amyloliquefaciens*, and barstar, its cognate intracellular inhibitor, as a system for investigation of protein folding mechanisms and protein-protein interactions was markedly facilitated several years ago by cloning and overexpression of the barnase gene, followed in a year by similar achievements for the barstar protein. Routinely, 100 mg quantities of either protein can be produced from a liter culture of *E. coli* bearing an appropriate genetically engineered plasmid. Efforts are now underway to characterize the structure of barstar and the 1:1 complex of the two proteins. A collaborative effort has been established with several European laboratories to advance study of the barnase:barstar system. The barnase crystal structure has been refined with 2.0 Å x-ray data. The structure of barstar in solution is well underway using 2-D NMR techniques in the laboratory of Dr. Jean Garnier in France, using the recombinant protein produced in our laboratory.

Recombinant barnase is identical to the native enzyme in all respects. Recombinant barstar differs from the native protein in having retained its N-terminal methionine. Both the native and recombinant proteins have variable degrees of oxidation of the two cysteinyl residues, although any of the forms of barstar are equally effective in the inhibition of barnase activity. The cysteines have been replaced singly or both by serines without effect on inhibitory activity. The seryl protein unfolds at a temperature about 20° lower than the native molecule with seemingly simpler equilibria. The three prolyl residues of barnase have been replaced by serine in an attempt to evaluate the possible role of proline isomerization in refolding. The proteins are active, with slightly reduced stability, and refold like the native molecule.

We have long been interested in the interactions between barnase and barstar. As one way of finding residues that are not important in this interaction, we have sequenced the genes for both proteins from three other, independent isolates of *B. amyloliquefaciens*. The most different has three

relatively conservative amino acid changes in each of the proteins. There are a number of silent base changes in the gene sequences. We are also using site directed mutagenesis to address these interactions. Twenty-five mutants (10 of barnase and 15 of barstar) are under investigation currently. Most are charges reversal mutants on the known surface of barnase or randomly in barstar. So far, two aspartyl residues (35 and 39) of barstar have been implicated in the interaction of the two proteins. More detailed study of the thermodynamics of the interaction may reveal other, more subtle, changes in stability of the complex. We use fluorescence measurements (the interaction of the two proteins quenches tryptophan fluorescence by about 50%) to monitor the interaction. We have found that addition of an excess of an inactive barnase mutant to a native barnase-barstar complex leads to the appearance enzyme activity over several hours. This provides a measure of the off rate for the complex and will be useful in detecting these subtle changes in interaction of the proteins.

Gene regulation in eukaryotic cells occurs in the context of chromatin, a complex of DNA, histones, and nonhistone proteins; this makes understanding of chromatin structure a prerequisite to the understanding of transcriptional regulation. Previous reports from this laboratory have documented the occurrence and, in several cases, the mechanism for sequence specific association of DNA with histone octamers, leading to what have been called positioned nucleosomes. An intriguing thought deriving from studies of positioned nucleosomes has been the possibility that positioning may be involved in the functional features of DNA sequence motifs. Several DNA segments, including promoters and replication origins, are found in chromatin at or near the terminus of a nucleosome core particle. Many years ago, we described the dynamic nature of the terminal 20 bp at each end of a core particle. We now ask whether nucleosome positioning serves a functional role. Our first attempt to address this question uses the core consensus ARS sequence in the TRP1ARS1 plasmid. The experiment involves creating mutant ARS's which will shift the consensus core DNA into a nucleosome. Deletions from 10 to 80 bp were made. Mapping of chromatin structure showed that indeed the deletions did move the ARS sequence further and further into the core particle as the length of the deletion increased. Copy number of the minichromosome was used as an indication of the efficacy of ARS function. Deletions of up to 50 bp were without effect on copy number which remained at about 40/cell. In striking contrast, deletions of 60 bp or more reduced copy number to one per cell. Placing a *cis*-acting element, in this case, a replication origin, in the region +/- 40 bp from the pseudodyad of the core particle markedly diminishes function of the element. Using the  $\alpha 2$  operator to move the nucleosome 50 bp away from the ARS (see below) rescues the -60 and -80 deletions; they are present in high copy number in  $\alpha$  cells. This is the first demonstration of an effect of placement of a DNA sequence relative to the nucleosome on function of that sequence *in vivo*.

## **CHROMATIN ORGANIZATION, TRANSCRIPTION AND REGULATORY FACTORS**

A set of striking observations have been made that, in conjunction with the above conclusion, suggest a novel mechanism for eukaryotic gene regulation. We have previously detailed results of study of the structure of yeast TRP1ARS1 plasmid chromatin. This 1453 bp episomal element contains two nuclease hypersensitive regions, one near the 5' end of the TRP1 gene and one near the 3' end of the gene and the functional ARS (replication origin) sequences. There are four unstable nucleosomes on the TRP1 gene. On a region of unknown function (UNF) between the ARS and the 5' end of the gene are three stable, precisely positioned nucleosomes. We have developed methods to purify the minichromosome using, initially, conventional biochemical methods and, more recently, protein-nucleic acid affinity. This year we have studied effects on chromatin structure and function of DNA sequences containing the operator for  $\alpha 2$ . This is a regulatory protein, synthesized in  $\alpha$  mating type cells but not in a mating type cells. It binds to a 31 bp sequence about 200 bp upstream of  $\alpha$ -specific genes and represses their transcription in  $\alpha$  haploids or  $\alpha/\alpha$  diploids.

Insertion of 80 or 300 bp of bacterial DNA into the nuclease hypersensitive region 5' of the TRP1 gene leads to randomly located nucleosomes on the majority of minichromosomes over UNF and the inserted DNA, although a fraction of the plasmids appear to have properly located nucleosomes I and II. When the  $\alpha 2$  operator is inserted between nucleosomes I and II, both these and nucleosome III are properly located. More surprisingly, when the  $\alpha 2$  operator is placed in the hypersensitive region, all three of these nucleosomes are properly located and, in addition, two new nucleosomes are formed

immediately adjacent to the  $\alpha 2$  site, one on each side. These results are obtained in  $\alpha$  haploids or  $a/\alpha$  diploids -- ordering of chromatin structure is not found in a cells, which do not synthesize the  $\alpha 2$  repressor protein. The simplest interpretation of these data is that  $\alpha 2$  interacts with histone octamers in some unknown way to form a domain with precisely defined chromatin structure. Interestingly, the  $\alpha 2$  site is about 200 bp upstream of the initiation codon for the five  $a$  specific genes that it represses. Organization of chromatin structure would place the RNA polymerase entry site in the middle of the nucleosome, just at the point where our studies of ARS indicate that *cis*-acting elements are nonfunctional. Although the TRP1 gene is not normally regulated by  $\alpha 2$ , its initiation codon is in about the same position relative to the  $\alpha 2$  operator in one of our constructs. Measurements of the amount of TRP1 mRNA showed it to be about 20 times higher in  $a$  cells, where nucleosome placement in this region is random, than in  $\alpha$  cells or  $a/\alpha$  diploids, where chromatin structure is highly organized. Thus, a eukaryotic repressor appears to turn off transcription by organizing a repressive chromatin structure. This mechanism is in strong contrast to those employed by prokaryotic repressors and helps to explain how eukaryotic regulatory proteins might act at a distance from the promoter.

Evidence suggesting that nucleosome positioning may be important in transcriptional regulation also has been obtained in studies of the *Xenopus* 5S rRNA gene. We reported previously that the presence of a nucleosome over the TFIIA intragenic promoter blocked initiation of transcription and the presence of a nucleosome downstream from the initiation site inhibited elongation by RNA polymerase III. Results from other laboratories suggest that RNA polymerase II, on the other hand, appears to be able to traverse nucleosomes during transcription of chromatin. We have used an inducible heat shock gene on a yeast plasmid to ask whether nucleosome dissociation occurs during this process. We assessed supercoil density on repressed and transcribed plasmids, using the thermal untwisting of DNA in yeast chromatin as a internal control for the activity of topoisomerase. We found that if nucleosome dissociation or disruption occurs during transcription, it must be reversible and transient; reestablishment of the native structure occurs within a few minutes.

We have also studied the chromatin organization of the yeast 5S rRNA gene inserted and amplified in the TRP1ARS1 plasmid. While the gene is not regulated, the 5S gene is one of the best studied transcriptional entities in biology; nevertheless, elucidation of the interplay between transcription factors IIIA, IIIB, IIIC and RNA polymerase III has not been possible. Indirect end label analyses were used to address the structure of the 5S gene in plasmid chromatin. A nuclease hypersensitive site is present within the 5S gene (this gene has a known intragenic promoter) and another site is located in the 5'-flanking region. Primer extension footprinting is currently being employed to correlate the hypersensitive sites with specific protein-DNA interactions in the putative transcription complex. The transcribed strand has striking DNase hypersensitive and nuclease resistant sites in the intragenic control region. The nontranscribed strand has a broad area of weakly protected sites in this region and both protected and hypersensitive sites in the 5' segment of the gene. These data suggest that other actors interact with the gene, *in vivo*, in addition to TFIIA, which binds to the intragenic control region *in vitro*. A mutant 5S "maxigene" which appears to not be active *in vivo* does not yield these patterns of nuclease cutting sites. We are now purifying RNA polymerase III to see if the minichromosome contains a functional transcription complex.

These studies, aimed at elucidating the structure of complexes containing previously identified *trans*-acting regulatory factors, provide a sound base for other investigations which have as their goal the identification of such *trans*-acting factors in more complex systems. One of these under development for the past three years is the search for proteins involved in the tissue and developmental stage specific regulation of human globin genes. The gene of particular interest is the  $\epsilon$ -globin gene, a member of the  $\beta$ -globin cluster expressed during embryonic life. K562 cells, derived from a human leukemia, express this gene. Nuclease hypersensitive sites are present in both 5' and 3' flanking regions when the gene is expressed; the 5' region contains a number of important sequence elements common to either most eukaryotic genes or to all globin genes and the 3' region contains sequences similar to described enhancer element sequences in human A- $\gamma$ - and  $\beta$ -globin and chicken  $\beta$ -globin genes.

Using mobility shift assays and footprinting, we have shown the presence of highly specific complexes formed with uninduced or induced K562 extracts and an oligonucleotide derived from

the 3' flanking hypersensitive region; these complexes were not found in extracts from non-erythroid cells. These erythroid specific complexes also formed with sequences from the human A- $\gamma$ - and  $\beta$ -globin enhancers. The factor is likely the human equivalent of EryF1 of chicken.

We have extended these studies by assaying the functional effects of the *cis*-acting sequences with a chloramphenicol acetyltransferase gene under control of  $\epsilon$  globin promoter electroporated into K562 cells. The 3' flanking region of the gene contains 14 repeats of the consensus binding sequence in about 2.5 kbp. It was divided into five fragments and each was tested for enhancer activity by placement 3' to the CAT gene. All fragments increased activity over the control, most about 5-fold, but one, which contained a sole copy of the sequence, was 10-fold higher. The 303 bp fragment is about 2 kbp 3' to the globin gene. Other enhancers also increased activity, the 3' chicken  $\beta$ -globin enhancer 100-fold and the human  $\gamma$ -globin enhancer 8-fold. Mutations of the three enhancer sequences are now being made and tested to assess the possible significance of the human *cis* element.

### DEVELOPMENTALLY REGULATED GENES

Another set of mammalian developmentally regulated genes of interest are those coding for the three proteins of the murine zona pellucida, an extracellular glycoalyx which surrounds the growing oocyte, functions to mediate species specific sperm interactions, prevents polyspermy, and protects the embryo prior to blastocyst implantation. We have reported previously cloning of cDNA and genomic clones for ZP3, the sperm receptor protein, characterization of the timing of expression of the gene, and its genomic organization. We have isolated full length cDNA and genomic clones for ZP2 also. Using DNA isolated from hamster-mouse somatic cell hybrids and from C57BL/6J-M. spretus interspecific backcross progeny, we have localized ZP3 to chromosome five, 9.2 +/- 2.9 cM distal to Gus and ZP2 to chromosome seven, 11.3 +/- 3.2 cM distal to g.

The ZP2 gene is made up of 18 exons, ranging in size from 45 to 190 bp with introns of 81-1490 bp. DNA sequences of flanking regions have been determined for 1.5 kbp at the 5' end and 0.8 kbp at the 3' end. A TATA box is present at -31 and a CCAAT box at -69. In contrast to the ZP3 gene, no repetitive elements are located in the flanking sequences or introns.

There are certain similarities in the mRNA's for ZP2 and ZP3. Both have very short untranslated segments, 30 and 29 nt at the 5' end and 32 and 16 nt at the 3' end for ZP2 and ZP3, respectively. Both are polyadenylated and synthesized exclusively in growing oocytes. Synthesis is undetectable in resting oocytes, peaks in 50  $\mu$ m oocytes and drops to very low levels in mature oocytes. Ovulated eggs contain significantly shorter message than growing oocytes, likely reflecting removal of the polyA tail. Determination of the predicted structures of the ZP2 and ZP3 proteins has revealed some similarities also. Both contain potential N-linked glycosylation sites as well as numerous serine and threonine residues as possible O-linked glycosylation sites. Each protein has a hydrophobic signal peptide with a peptidase recognition site. Secondary structure prediction algorithms show some similarities between the two proteins, the most interesting of which is a very hydrophobic segment (like those usually thought to be involved in trans-membrane domains) near the C terminus. Two five amino acid segments are present in both proteins; TLGSE at about 45 amino acids from the N termini and VSLPQ at the N terminus of ZP2 and the C terminus of ZP3. These identities are highly unlikely to occur by chance; their role in the postulated crosslinking of the ZP2-ZP3 matrix by ZP1 is provocative.

Having two of the three zona protein genes sequenced provided an opportunity to begin a search for *cis* elements which might be involved in their coordinate, tissue specific expression. Within the first 250 bp 5' to the transcription start site, three 8-12 bp regions of 80-88% homology were detected in the two genes. We have placed 840 bp of the 5' flanking region of ZP3 upstream of  $\beta$ -galactosidase on a circular plasmid as a reporter construct for initial screening for the possible significance of these homologous regions in transcription. Microinjection of the DNA into growing oocytes revealed a strong  $\beta$ -galactosidase activity. Deletion analysis of the region showed that about 200 bp, interestingly including the homologous regions, was necessary for  $\beta$ -galactosidase expression in oocytes. Control experiments using microinjection into fibroblasts suggested the presence of a

negative regulatory element in addition to the presumed positive elements suggested from the oocyte injection study.

We previously had shown a high degree of conservation of the ZP3 gene among mammals by Southern blot zoo hybridization. We have now isolated a genomic clone for a large portion of the human ZP3 gene and determined its sequence. The sequence in hand codes for exons 1-5 of ZP3. Similarity to the murine DNA and protein sequence is high, 82% in amino acid sequence for exons 2-5. Interestingly, the similarity in exon 1 is lower, 66%, including a 12 amino acid segment which is quite unrelated. The possible significance of the differences in exon 1 (or the other, as yet undetermined exons) in species specific sperm receptor activity remains to be determined. There are somewhat intriguing, but no striking, similarities in sequence in the 5' flanking regions of the murine and human ZP3 genes. Although the possible significance of the repeated elements flanking the murine gene is unknown, the human gene also has repeated elements in this region. The repeats, Alu in human and B1 in mouse, have a high degree of homology. In consideration of the potential significance of these, it should be remembered that no repeat elements are found around the murine ZP2 gene.

We have begun experiments whose goal is study of the role of zona protein expression in oocyte development and ovarian morphogenesis. Two methodologic approaches are being employed. In one, we hope to use diphtheria toxin A chain genes under ZP3 promoter control to create a null mutation in transgenic mice. Secondly, we have inserted a stop codon into the first exon of the ZP3 gene and attempt to obtain homologous recombination of this mutated gene into embryonic stem cells for creation of transgenic animals. Experiments thus far have shown integration of the stop codon containing fragment into NIH 3T3 fibroblasts after microinjection in 7/17 pools, each containing 100 microinjected cells. Site specific integration was not observed in these preliminary experiments.

In a much smaller organism, yeast, we use genetics to study the mechanism of regulation of a particular gene under control of the MAT locus (mating type). The products of the MAT locus are regulators of large batteries of genes which specify cell type. Several of the MAT locus proteins are DNA binding molecules which regulate transcription of target genes. We study a strain of yeast which secretes amylase (STA1) under control of the MAT locus. The enzyme is secreted by haploid cells and by cells homozygous at MAT, but not by heterozygous MAT diploids. We constructed a complete deletion of MAT $\alpha$ 2, one of the two known transcriptional units of MAT $\alpha$  and showed that this deletion does not affect MAT regulation of amylase secretion. Deletion of MAT $\alpha$ 1, on the other hand, abolished mating type control of glucoamylase secretion. Extending these studies, we have shown that regulation of STA1 occurs at the level of RNA accumulation, STA1 is glucose-repressible, and STA1 expression is induced during sporulation, together with that of a homologous, intracellular amylase SGA. The two amylases are regulated differently, a diploid strain with a MAT $\alpha$ 1 deletion expresses STA1 in sporulation medium but does not express SGA. We have identified a novel haploid specific gene by using the STA1 signal sequence as a probe for RNA blots from yeast strains which lack the STA1 gene. The 4 kb message appears to be regulated in exactly analogous fashion to STA1. Finally, we have introduced a MAT $\alpha$  gene into a MAT $\alpha$  cell, where it represses glucoamylase synthesis. Mutagenesis of the MAT $\alpha$ 1 gene is being used to identify protein domains responsible for interaction of MAT $\alpha$ 1 and MAT $\alpha$ 2 to form the heterodimeric repressor of haploid-specific genes.

Another yeast gene expressed during sporulation, HSP82, is also under study. This gene contains a sporulation-inducible nuclease hypersensitive site several hundred base pairs 5' to the transcription unit; this site is replete with DNA sequences characteristic of binding sites for a number of known *trans*-acting factors. We have constructed the appropriate supersporulating yeast strain with genetic markers allowing gene replacement and selection. Conditions and media for effective sporulation have been developed. After a number of difficulties with polymerase chain reaction methodology for making the initial deletion mutants of the hypersensitive region (due to an incorrect sequence for the gene fragment, determined by others), construction of the mutants and transformation of yeast is well underway.

A developmental system under study that also leads to spores is *Dictyostelium discoideum*, albeit in a much more complicated developmental pathway than *Saccharomyces cerevisiae*. We have defined a number of genes which are differentially expressed during development of this slime mold, and

defined several mechanisms (or pathways) by which such regulation occurs, including multiple mechanisms involving the classic second messenger, cAMP. We have now determined that another class of genes is regulated by a neo-classical second messenger pathway,  $\text{Ca}^{++}$ , 1,4,5 inositol trisphosphate ( $\text{IP}_3$ ), and diacylglycerol (DAG). Addition of  $\text{IP}_3$  and/or DAG can bypass the usual requirement of extracellular cAMP for expression of these genes, suggesting that induction of transcription derives from receptor activation of phospholipase C.  $\text{IP}_3$  mobilizes  $\text{Ca}^{++}$  and can activate guanylate cyclase to increase cGMP concentrations in *Dictyostelium*. Thus, the several protein kinases activated in these diverse second messenger systems may be pivotal in developmentally regulated gene expression in *D. discoideum*; as in a few other systems, phosphorylation and dephosphorylation of *trans*-acting factors may be the key to regulation of transcriptional activity.

## SIGNAL TRANSDUCTION AND HORMONE ACTION

The studies summarized in the previous paragraph are an obvious transition from investigations of genes involved in development to the work in LCDB which is concerned with signal transduction -- how environmental information is translated into cytoplasmic and nuclear adaptation by the cell. Recent investigations in the laboratory have defined several of the players in this drama in *Dictyostelium*. Last year, in collaboration with a research group at Johns Hopkins, we isolated a gene for the primary cAMP cell surface receptor. It is a typical receptor with seven transmembrane domains and a long cytoplasmic tail at the C-terminal end of the predicted protein, containing multiple seryl residues likely involved in the phosphorylation induced down regulation of the receptor during cell-cell signalling in development. We have now shown that disruption of the normal pattern of expression of the receptor with anti-sense RNA totally blocks normal development of the organism, confirming the pivotal role of this protein in normal growth of *Dictyostelium*. In addition, using the cloned gene as a probe, we have identified two additional, related genes. Sequence analysis of these genes reveals a very high degree of homology with the first identified cAMP receptor, suggesting that there is a family of cAMP receptor proteins in this organism. The transmembrane domains are nearly identical for all three proteins and an intron is positioned in exactly the same site for all three genes. Differences between the three proteins are greatest in the intracellular C-terminal region. The temporal and spatial locations of expression of the three genes during development are strikingly different, a tantalizing finding which suggests complicated, but addressable, mechanisms of regulation of gene expression during development by cAMP, a paracrine hormone for *D. discoideum*.

Some (or all) cell surface receptors interact with a heteromultimeric complex of proteins collectively called G-proteins, based on their initial identification by binding guanine nucleotides. We have isolated two genes, SAS1 and SAS2, which may code for *Dictyostelium* G-proteins. The predicted structure of the proteins shows lengths of about 200 amino acids with 90% sequence identity to each other and a high degree of conservation of sequences in a domain thought to be involved in interaction of proteins with GTP. The gene products are closely related to two essential yeast genes, YPT1 and SEC4; these genes produce proteins which are involved in membrane trafficking, suggesting a possible similar role in *D. discoideum*. We have not yet been able to complement yeast mutations in YPT1 and SEC4 with exogenous SAS genes. The developmental expression of the SAS genes has been defined temporally.

Signal transduction has also long been an interest of members of LCDB who study the mechanisms whereby hormones regulate cellular metabolism, particularly in isolated adipocytes, a model for molecular endocrinology established by Dr. Martin Rodbell, a former member of the laboratory. Recently, improvements in the isolation of rat fat cells have led to a preparation which is highly reproducible in its metabolic characteristics and accurately mirrors the *in vivo* situation. Current interests involve the regulation of lipolysis by hormones which stimulate adenylate cyclase and by insulin.

We have examined the phosphorylation state of a variety of cellular proteins under different physiological conditions. After loading cells with  $^{32}\text{P}_i$ , phosphorylation of a 65 kD protein and a 62 kD protein was shown to be altered in opposite fashion when cells were exposed to insulin. Modification of the 62 kD protein increased while that of the 65 kD protein diminished.

Phosphorylation of the 65 kD protein was increased when the activity of cAMP-dependent protein kinase was stimulated. We have now purified and characterized these two species with the surprising finding that they are apparently the same polypeptide, whose electrophoretic mobility differs depending on level of phosphorylation or conformational changes due to the modification. This protein is located at the surface of the lipid droplet in adipocytes and appears to occur uniquely in these fat cells. Alterations in the modification state of the protein by hormones known to affect lipolysis, together with its physical location on the fat droplet, suggest a possible role for the protein in fundamental features of metabolism of energy storage compounds. The concerted effects of hormones on the modification state of this major phosphoprotein is consistent with our previous interpretation of the possible mechanism of cAMP-independent inhibition of lipolysis by insulin.

That is, the presumed modification state of hormone sensitive lipase (HSL) follows that of the 65 kD protein. We have now purified HSL and produced a polyclonal antiserum against the protein in rabbits. This enzyme is the rate limiting activity in lipolysis. Surprisingly, phosphorylation of the isolated enzyme *in vitro* does not lead to increases in lipolytic activity; phosphorylation *in vivo* leads to marked increases in lipolysis, in contrast. The explanation for this apparent paradox seems to lie in the subcellular localization of HSL *in vivo*. Using the antibody to HSL, we have shown that lipolytic hormones, which increase phosphorylation of HSL, also induce translocation of the enzyme from cytosol to the surface of the fat droplet. Both Western blots of proteins isolated after subcellular fractionation and fluorescent antibody *in situ* localization in cultured fat cells demonstrate this translocation. At this point, we would speculate that increased lipolysis in adipocytes after hormonal stimulation is a result of the movement of HSL from one subcellular compartment to another, bringing enzyme and substrate into proximity.

Insulin has been thought, by some, to lead to increases in protein kinase C activity in adipocytes, although this has been a matter of contention. The possible resolution of this situation has been made recently in our laboratory by the finding that translocation of this enzyme also occurs when fat cells are treated with insulin. Low concentrations of the hormone lead to movement of the kinase to plasma membranes. As with so many advances in understanding cell biology, this observation is the direct outcome of a technical development, specifically a very sensitive protein kinase assay which allows dilution of cell extracts to the point where the activity of a potent, endogenous inhibitor of protein kinase C is reduced sufficiently that enzymatic activity can be measured. All these studies of signal transduction in the well studied adipocyte complement in bidirectional fashion the studies of paracrine hormonal regulation of differentiation in *D. discoideum*, for the benefit of both research areas.

An interest in signal transduction and mechanisms of differentiation that lead to hormonal responsiveness has generated a system under investigation in LCDB for several years. MDCK cells lose responsiveness to glucagon when transformed by Harvey MSV. A number of small molecules, most noticeably prostaglandins, lead to a return of hormone responsiveness when cells are cultured in their presence. Coordinated with the development of glucagon responsiveness is a decrease in the expression of p21, the product of the ras oncogene. Our previous work suggested that cAMP was likely to be the primary signal in development of hormone responsiveness. We have found that subsequent to increases in cAMP, but antecedent to the return of glucagon sensitivity, PGE<sub>2</sub> decreases the concentration of IP<sub>3</sub> in transformed MDCK cells. Phorbol esters block the induction of glucagon responsiveness and also block the alteration in content of IP<sub>3</sub>, suggesting that alterations in metabolism of inositol phosphates by cAMP might be a causal event in development of hormone responsiveness.

In order to study the possible relationship of changes in ras expression to this process, we made transfected NIH 3T3 and MDCK cell lines containing a ras oncogene under control of the MMTV steroid-inducible promoter. These cells exhibit a decrease in EGF binding (probably loss of a class of high affinity receptors) when transcription of the oncogene is induced by dexamethasone; hormone responsiveness to glucagon or  $\beta$ -adrenergic hormones is unaffected by induction of ras p21 expression. Concentrations of diacylglycerol also increase when ras is expressed. Based on the observations of others that (i) diacylglycerol is an activator of protein kinase C and (ii) phorbol esters lead to a decrease in high affinity EGF receptors, we speculate that ras may activate protein kinase C and thereby lead to the decrease in EGF binding in these cell lines. Biochemical analysis of the

EGF receptor in these cells is difficult, due to the low number of receptors per cell. To circumvent this problem, we have transfected a pituitary cell line, GH<sub>3</sub>, which has a high content of EGF receptors, with a plasmid containing the v-Ha-ras gene under control of a dexamethasone inducible promoter.

## **LIPID METABOLISM**

In addition to the direct study of mechanisms of hormone action on fat cells, others in the laboratory study genetic defects in lipid metabolism, enzymes involved in lipolysis and clinical manifestations of lipid disorders. Mice born with combined lipase deficiency (cld/cld, a recessive mutation in the T/t complex of chromosome 17) develop extreme hyperchylomicronemia and die within three days if allowed to suckle. They have very low levels (<5% of normal) of lipoprotein and hepatic lipase activities (the genes for these enzymes are located on chromosomes 9 and 11, respectively), their tissues are virtually devoid of fat, and 95% are tailless. Studies *in vivo* showed that brown adipose and other tissues of the cld/cld mice synthesized normal sized lipoprotein lipase protein but the enzyme was inactive and not transferred to capillaries, the normal site of action of the enzyme.

We found that cells cultured from brown adipose tissue of 1-day old cld/cld and unaffected mice readily differentiated into brown adipocytes. Using this manipulatable tissue culture system, we have studied aspects of the metabolism of affected cells and processing of lipase. Unaffected cells synthesize a dimeric, active, secretable lipoprotein lipase (Mr 58000) with two complex oligosaccharide chains. The cld/cld cells synthesize a partially dimeric, inactive, nonsecretable lipase (Mr 56000) which contains only high mannose type oligosaccharides. The cld/cld cells contain twice as much immunoreactive lipase as normals, but less than 5% the enzymatic activity. The size of mRNA for lipoprotein lipase in cld/cld cells is the same as that in unaffected cells; the level of message was higher in the mice bearing the genetic lesion. Southern blot analysis of the lipase gene in cld/cld mice showed no restriction length polymorphisms (16 enzymes), relative to normal cells.

Immunofluorescent localization was used to follow the processing and secretion pathway of lipase in mutant and control cells. Normal cells secreted lipase rapidly; the antigen was undetectable unless cells were treated with monensin to block intracellular transport, in which case the lipase accumulated in the Golgi. In cld/cld cells, immunologic reactivity was observed in a diffuse, reticular pattern, suggesting that transport of lipoprotein lipase was arrested at the stage of transfer from the endoplasmic reticulum to Golgi. This observation, together with the apparent defect in processing of high mannose type oligosaccharides in cld/cld cells, led to studies of unaffected cells treated with swainsonine, an inhibitor of mannosidase II. Adipocytes treated with this drug secreted an active lipoprotein lipase, containing unprocessed high mannose type oligosaccharide chains, indicating that processing of these oligosaccharides in Golgi is not necessary for activity or secretion of lipoprotein lipase.

Similarly, a study of hormonal responsiveness of cld/cld adipocytes, compared with normal fat cells, has revealed only minor differences in both incorporation of labeled glucose into triacylglycerol stimulated by insulin and in lipolysis stimulated by isoproterenol. In all cases, both kinetics and dose-response curves were closely similar. Thus there appears to be no lesion in either lipogenesis or hormone sensitive lipolysis in cld/cld cells. At this point, the studies we have carried out have eliminated a number of possible mechanisms for the defect in lipase activities in the cld/cld mouse. Whether processing of oligosaccharides in the endoplasmic reticulum, ER to Golgi transfer, or yet some other process is affected by the mutation remains to be determined.

A possible role for lipoprotein lipase in the cachexia of tumor patients has been suggested by studies in LCDB in the past year. Some cachectic patients have low plasma concentrations of tumor necrosis factor (TNF), also called cachectin, and high concentrations of interleukin 6 (IL-6). TNF increases the production of IL-6 in animal studies. We could demonstrate that IL-6 decreases lipoprotein lipase mRNA and enzymatic activity in both freshly isolated adipocytes and fat cells in tissue culture. Unexpectedly, we have found that isolated fat cells are highly active in synthesis of IL-6 mRNA and the factor itself, suggesting a novel role for adipose tissue in production of hormones and growth factors that may be involved in a spectrum of inflammatory and/or metabolic processes.

We also study lipid metabolism in human diseases such as Type C Niemann-Pick (NP-C) disease, an autosomal recessive neurovisceral lipid storage disorder. Fibroblasts derived from patients with NP-C accumulate excessive amounts of unesterified cholesterol when incubated with LDL. We have used fluorescent probes to determine the sites of cholesterol accumulation. Exogenous LDL-cholesterol is transferred via lysosomes to Golgi in both normal and NP-C fibroblasts over periods from 2-24 hours. The Golgi is also involved in transport of ceramide, a precursor of sphingomyelin and glucosylceramide. Using fluorescent ceramide, we have shown that LDL loading leads to enhanced ceramide in Golgi stacks in both normal and NP-C fibroblasts. Normal fibroblasts show a similar response when preincubated with cholesterol precursors, suggesting that endogenously synthesized and exogenous (LDL) cholesterol follow similar intracellular pathways and suggesting the possibility that cholesterol may be the lipid which affects ceramide accumulation in Golgi membranes. The role of the Golgi in the altered lipid storage in Niemann-Pick Type C disease is under continuing investigation.

### **BIOTECHNOLOGY AND APPLIED BIOCHEMISTRY**

Several areas of research in LCDB are closer to bringing the results of basic investigations to use in clinical situations or for production of materials for other research. I have grouped them together in this section of the report, even though their subject matter is not logically coherent.

The Biotechnology Unit is a service facility for all of NIH, in addition to serving in research and development of biotechnological methodology, particularly production of microbial, fungal and tissue culture cells and initial large-scale processing of biologicals. During the past year over 120 fermentations in volumes from 10 to 300 liters were performed. Mammalian tissue culture cells were grown for various groups at NIH in volumes up to 50 liters. A number of fermentations were for production of toxins for potential clinical applications; notable were growth of *Bordetella pertussis* for toxin purification and toxoid preparation to be used as a more efficacious whooping cough vaccine, growth of *Pseudomonas aeruginosa* for isolation of exotoxin to be used in development of antineoplastic directed cytotoxic agents and growth of *Vibrio cholera* for toxin production for vaccine development. Other studies concentrated on improvement of yields of toxins and biomass; yields of bacterial cells up to 100 g/L have been obtained recently.

Noted before were studies of ZP3, the murine sperm receptor. We had shown previously that antibody to ZP3 could be used to passively immunize mice against conception in an effective, but reversible, fashion. We have used several strategies to try to develop an active contraceptive vaccine. First, we cloned short segments of ZP3 cDNA into an expression vector, localized the clones that expressed the epitope recognized by a monoclonal antibody known to confer passive immunity against contraception, and determined the amino acid sequence of the epitope. This seven amino acid sequence (embedded in a 16 amino acid peptide) was then synthesized, coupled to a carrier protein and used to immunize mice. Antibody to the complex was produced in female mice. Titers were sufficiently high that antibody could be found bound to the zonae surrounding growing oocytes in the murine ovaries. Pregnancy was prevented during a nine-month period in 75% of the treated animals. We have extended these studies by making a ZP3-vaccinia virus recombinant and vaccinating mice with the chimaeric virus. Anti-ZP3 antibodies were produced by vaccinated females and mating studies are currently underway to see if this approach to long-term contraception works also. Given the close relatedness of the zona genes from a variety of mammalian species, the potential to extend such studies to other animals is high.

Studies of dihydrofolate reductase, the target enzyme for methotrexate, an antimetabolite of high clinical importance in treatment of cancer and autoimmune diseases, have been carried out in LCDB for more than two decades. Collaborative investigations of (i) the structure and (ii) interactions with drug derivatives of the enzyme from several vertebrate sources continue.

Studies of barnase activity have been facilitated by development of a novel assay using a fluorescent substrate, polyethenoadenosine. Four kinetic steps are observed in hydrolysis, the first three being hydrolysis to oligomers, dimers and finally monomers; these all involve transesterification to 2'-3'

cyclic phosphates. The last step, slower than the others, is hydrolysis of the cyclic monomer. More detailed investigation of engineered mutants should be possible with this assay method in hand. Making these (and other) mutants has become easier and faster with development of a novel extension of the polymerase chain reaction mutagenesis protocol. We now can produce mutants of expression plasmids directly *in vitro* in as little as three days. Another extension of PCR methodology was made to study the amounts of low level mRNAs (specifically, for the SAS genes described above). Primers homologous to all members of a gene family are used to amplify mRNA. Specific gene sequences can then be detected by restriction enzyme polymorphisms or gene specific oligonucleotide probes.

DEPARTMENT OF HEALTH AND HUMAN SERVICES • PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 15004-14 LCDB
PERIOD COVERED <b>October 1, 1988 to September 30, 1989</b>		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <b>Modulation of Hormone-Responsive Systems by ras Oncogene Product</b>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.: Others:	Michael C. Lin Yvonne Wu Beatrix White	Research Chemist Senior Staff Fellow IRTA Fellow  LCDB:NIDDK LCDB:NIDDK LCDB:NIDDK
COOPERATING UNITS (if any)  Thomas Shih, C:FCRF; Eugenio Santos, LLM:NIAID; Gordon Guroff, NICHD		
LAB/BRANCH Laboratory of Cellular and Developmental Biology		
SECTION Developmental Biochemistry Section		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 3.2	PROFESSIONAL: 2.4	OTHER: 0.8
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unabbreviated type. Do not exceed the space provided.) <p>             The goal of our research is to understand the modulation of signal transduction mechanisms during the expression of a well-defined oncogene. We had previously found that ras transformation of MDCK cells caused a selective loss of glucagon receptors and this loss can be restored by treatment of cells with PGE<sub>2</sub>. The effect of PGE<sub>2</sub> seems to be mediated by cyclic AMP. We have now found that, subsequent to the elevation of cyclic AMP, but preceding the appearance of glucagon sensitivity, PGE<sub>2</sub> decreased the level of inositol 1,4,5-trisphosphate in the transformed MDCK cells. Both effects of PGE<sub>2</sub> on IP<sub>3</sub> and on the induction of hormone sensitivity were blocked by phorbol ester. We conclude that the perturbation of the IP<sub>3</sub> pathway by cyclic AMP is likely to be causally related to the induction of glucagon response. In order to examine the causal relationship between the expression of ras gene and the various changes of signal transduction more closely, we have transfected cells with plasmid containing inducible ras oncogenes to allow more precise control of p21 production. Using transfected 3T3 cells (obtained from others) and MDCK cells, we found that cyclic AMP-mediated hormone sensitivity is unaltered even when ras is fully expressed. In contrast, EGF receptors are rapidly desensitized, due to a loss of high affinity sites, when ras is expressed. Since diacylglycerol is also elevated when p21 is induced by dexamethasone, the desensitization of EGF receptors is likely caused by an activation of protein kinase. We are now screening the transfected GH<sub>3</sub> cell line, which possesses high density EGF receptors, for clones expressing inducible ras, so that we can study the mechanism responsible for the desensitization of EGF receptors by protein kinase C. We have found a rapid increase in transfected 3T3 cells and a rapid decrease of PGE<sub>2</sub> production in transfected MDCK cells, upon the expression of ras gene. This is consistent with previous findings using virally transformed cells.           </p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 15005 -14LCD
<b>PERIOD COVERED</b>		
October 1, 1988 to September 30, 1989		
<b>TITLE OF PROJECT</b> (80 characters or less. Title must fit on one line between the borders.)		
Regulation of Adipocyte Metabolism		
<b>PRINCIPAL INVESTIGATOR</b> (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.:	Constantine Londos	Research Chemist LCDB:NIDDK
Others:	John J. Egan	Staff Fellow LCDB:NIDDK
	Andrew S. Greenberg	Senior Staff Fellow LCDB:NIDDK
	Soraya Naghshineh	Senior Staff Fellow LCDB:NIDDK
	Nira B. Garty	Visiting Fellow LCDB:NIDDK
	Sheree A. Wek	Biologist LCDB:NIDDK
<b>COOPERATING UNITS</b> (if any)		
I.A. Simpson, S.W. Cushman, and J. Saltis, MCBEB:NIDDK; M. Moos, K.B. Seamon, CDB:DB; A.R. Kimmel, C.L. Saxe, J. Blanchette-Mackie, R.O. Scow, C. Mateo, LCDB:NIDDK; D.M. Jablons, DCT:NCI; K.P. Huang, ERBB:NICHHD		
<b>LAB/BRANCH</b>		
Laboratory of Cellular and Developmental Biology		
<b>SECTION</b>		
Membrane Regulation Section		
<b>INSTITUTE AND LOCATION</b>		
NIDDK, NIH, Bethesda, MD 20892		
<b>TOTAL MAN-YEARS:</b>	<b>PROFESSIONAL:</b>	<b>OTHER:</b>
5.0	4.5	.5
<b>CHECK APPROPRIATE BOXES)</b>		
<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither
<input type="checkbox"/> (a1) Minors		X
<input type="checkbox"/> (a2) Interviews		
<b>SUMMARY OF WORK</b> (Use standard unexpanded type. Do not exceed the space provided.)		
<p>We have examined various aspects of hormonal control of adipocyte metabolism with isolated rat adipocytes and with cultured white and brown fat cells as model systems. The following findings are summarized: (A) Previously, we found two hormonally regulated phosphoproteins, by far the most abundant phosphoproteins in fat cells, associated with the lipid storage droplet. Further study reveals that these two species represent different phosphorylation states of a single polypeptide. Moreover, this protein appears to be found only in adipocytes, and its location at the surface of the lipid droplet may be related to the following findings. (B) Hormone-sensitive lipase (HSL), the rate-limiting enzyme of lipolysis, has been purified in this laboratory, and phosphorylation of the enzyme <u>in vitro</u> does not lead to increased substrate hydrolysis, despite the fact that phosphorylation <u>in vivo</u> leads to greatly increased lipolysis. The basis for this apparent discrepancy is our finding that upon phosphorylation <u>in vivo</u>, the enzyme translocates from the cytosol to the surface of the lipid storage droplet. This change in location of the enzyme has been confirmed both by Western blotting of subcellular fractions with anti-HSL antibody and by visualization of the enzyme in cultured cells with fluorescent probes. (C) The question of insulin-stimulated activation of protein kinase C in adipocytes has been under dispute. With a new, extremely sensitive protein kinase assay method, we have found that low concentrations of insulin rapidly translocates protein kinase C to plasma membranes of isolated adipocytes. Related findings are that vasopressin and oxytocin also stimulate PKC in these membranes, and that adipocytes are highly enriched in protein kinase C, the detection of which is hampered by the presence of a highly potent, yet unidentified inhibitor of the enzyme. (D) Cachectic patients exhibit high concentrations of plasma Interleukin-6 (IL-6). We have found that IL-6 acts directly on both freshly isolated adipocytes and on cultured 3T3-L1 adipocytes to decrease lipoprotein lipase activity. Isolated adipocytes also produce high amounts of IL-6.</p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER  Z01 DK 15100-19 LCDB																		
PERIOD COVERED October 1, 1988 to September 30, 1989																				
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Protein Nucleic Acid Interactions: Chromatin Structure and Function																				
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">P.I.: R.T. Simpson</td> <td style="width: 33%;">Laboratory Chief</td> <td style="width: 33%;">LCDB:NIDDK</td> </tr> <tr> <td>Others: A. Dranginis</td> <td>Senior Staff Fellow</td> <td>LCDB:NIDDK</td> </tr> <tr> <td>R. Morse</td> <td>Senior Staff Fellow</td> <td>LCDB:NIDDK</td> </tr> <tr> <td>R. Parker</td> <td>Senior Staff Fellow</td> <td>LCDB:NIDDK</td> </tr> <tr> <td>S.Y. Roth</td> <td>Senior Staff Fellow</td> <td>LCDB:NIDDK</td> </tr> <tr> <td>C. Szent-Gyorgyi</td> <td>Senior Staff Fellow</td> <td>LCDB:NIDDK</td> </tr> </table>			P.I.: R.T. Simpson	Laboratory Chief	LCDB:NIDDK	Others: A. Dranginis	Senior Staff Fellow	LCDB:NIDDK	R. Morse	Senior Staff Fellow	LCDB:NIDDK	R. Parker	Senior Staff Fellow	LCDB:NIDDK	S.Y. Roth	Senior Staff Fellow	LCDB:NIDDK	C. Szent-Gyorgyi	Senior Staff Fellow	LCDB:NIDDK
P.I.: R.T. Simpson	Laboratory Chief	LCDB:NIDDK																		
Others: A. Dranginis	Senior Staff Fellow	LCDB:NIDDK																		
R. Morse	Senior Staff Fellow	LCDB:NIDDK																		
R. Parker	Senior Staff Fellow	LCDB:NIDDK																		
S.Y. Roth	Senior Staff Fellow	LCDB:NIDDK																		
C. Szent-Gyorgyi	Senior Staff Fellow	LCDB:NIDDK																		
COOPERATING UNITS (if any) T. Richmond, ETH, Zurich, Switzerland (foreign) D.S. Pederson, University of Vermont Medical School K.E. van Holde, Oregon State University																				
LAB/BRANCH Laboratory of Cellular and Developmental Biology																				
SECTION Developmental Biochemistry Section																				
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892																				
TOTAL MAN-YEARS: 6.0	PROFESSIONAL: 6.0	OTHER: 0.0																		
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews																				
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>             This project uses a variety of approaches to address fundamental questions relating to chromatin structure, transcription, and replication. In the past year, we have, for the first time, demonstrated an effect on the function of a cis-acting DNA element of its position in a nucleosome, using deletions of the TRP1ARS1 yeast plasmid and studying the efficiency of the replication origin. Mutants in which the ARS core sequence is located within 30-40 bp of the core particle pseudodyad are markedly impaired in replication. The significance of nucleosome positioning on function is reinforced by studies of yeast plasmids containing promoter elements under negative regulation by alpha 2 in yeast alpha-cells. Binding of alpha 2 to this element organizes chromatin structure; two positioned nucleosomes are placed on either side of the trans-acting factor when it is present and binds to its DNA element. In contrast, in cells lacking alpha 2, chromatin structure of the region is totally random. Functional studies of transcription of the Xenopus 5S rRNA gene in oocyte extracts have demonstrated that nucleosomes block elongation by RNA polymerase III, in contrast to results <u>in vitro</u> for other polymerases. A yeast 5S rRNA gene in a multicopy plasmid has a chromatin structure which suggests the interactions of multiple regulatory factors, in addition to that of the characterized TFIID. We have addressed the question of possible nucleosome dissociation during transcription by polymerase II <u>in vivo</u> using the yeast HSP26 gene cloned in a plasmid. We find no stable disruption of nucleosomes during transcription. We have shown that secretion of a haploid specific amylase is not, as previously suggested by others, under control of the yeast mating type protein a2. Other continuing studies include the chromatin structure of a possible sporulation specific promoter for the HSP80 gene, high resolution X-ray structure of the core particle, and higher order chromatin structure.           </p>																				

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 15102-2 9LCDB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Study of Ribonuclease and its Inhibitor from *Bacillus amyloliquefaciens*

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Robert W. Hartley

Others: Peter FitzGerald

## COOPERATING UNITS (if any)

C. Hill, Dept. of Chem., UCLA; J. Garnier, Protein Engineering Unit, Biotechnology INRA, Jouy-en-Josas, France; A.R. Fersht, Chem. Dept., Cambridge U.; N. Vasantha, Experimental Station, I.E. de Pont de Nemours, Wilmington

## LAB/BRANCH

Laboratory of Cellular and Developmental Biology

## SECTION

Developmental Chemistry Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2

## PROFESSIONAL:

2

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Two proteins, barnase, the extracellular ribonuclease of *Bacillus amyloliquefaciens*, and barstar, its intracellular inhibitor, are used as a model system for the study of protein folding and protein-protein interactions. Barnase is one of an homologous group of ribonucleases occurring in both prokaryotes and eukaryotes.

Recombinant DNA techniques are being applied with three major aims: (1) to facilitate production of wild type and mutant proteins; (2) to examine the structural and control sequences of the genes; and (3) to make specific changes in the sequences to test theories of folding and probe the barnase-barstar interaction.

The lethal effect of the cloned wild type barnase gene can be repressed by expression of the barstar gene on the same plasmid. *E. coli* plasmid vectors have been devised for both proteins and both can now be obtained essentially pure in 100 mg quantities. DNA and amino acid sequences are known for both and the x-ray structure of barnase has been refined to 2.0 Å. The structures of both proteins in solution are being studied by 2-D NMR. Several new techniques have been devised, including an improved method of introducing directed mutations using the polymerase chain reaction (PCR), a method for detecting barstar activity in bacterial colonies on agar plates, use of a synthetic fluorescent substrate for studying hydrolysis kinetics and the kinetics and equilibria of the reactions between the wild type and mutant proteins and also the use of polyacrylamide gel electrophoresis to observe binding between barnases and barstars.

Significant differences in both DNA and protein sequences have been observed in both barnase and barstar genes cloned from four independently isolated strains of *B. amyloliquefaciens*. A complete set of charge-reversal mutants (e.g., Glu to Lys) of barstar have been prepared and two residues involved in the reaction with barnase identified. A set of mutants over the (known) surface of barnase has also been prepared.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 15200-29 LCDB						
PERIOD COVERED <b>October 1, 1988 to September 30, 1989</b>								
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <b>Studies on Folic Acid (Dihydrofolate Reductase) and Vitamin A</b>								
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%; vertical-align: top;">P.I.: Bernard T. Kaufman</td> <td style="width: 33%; vertical-align: top;">Research Chemist</td> <td style="width: 33%; vertical-align: top;">LDCB:NIDDK</td> </tr> <tr> <td style="vertical-align: top;">Others: John Bieri</td> <td style="vertical-align: top;">Scientist Emeritus</td> <td style="vertical-align: top;">LCDB:NIDDK</td> </tr> </table>			P.I.: Bernard T. Kaufman	Research Chemist	LDCB:NIDDK	Others: John Bieri	Scientist Emeritus	LCDB:NIDDK
P.I.: Bernard T. Kaufman	Research Chemist	LDCB:NIDDK						
Others: John Bieri	Scientist Emeritus	LCDB:NIDDK						
COOPERATING UNITS (if any) Department of Chemistry, University of California, San Diego Nutrition Research Center, USDA, Beltsville, MD								
LAB/BRANCH Laboratory of Cellular and Developmental Biology								
SECTION Nutritional Biochemistry Section								
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892								
TOTAL MAN-YEARS: 2	PROFESSIONAL: 2	OTHER:						
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews								
SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.) <p>             Methotrexate (MTX) is a key drug in the treatment of a variety of diseases, however, hepatocellular necrosis, fibrosis and cirrhosis are major problems associated with MTX therapy. Since the mechanism of these toxic effects is not completely understood, we continue our studies on dihydrofolate reductase (DHFR) of hepatic origin -- the target for MTX action. In addition, DHFRs of animal cells and tissues have the unique ability to have their catalytic activity markedly increased by a variety of agents which are usually deleterious to enzyme activity. The chicken liver reductase (CLDHFR) is the only animal DHFR whose three-dimensional structure is known in sufficient detail to consider structure-function relations. In addition, CLDHFR exhibits the most dramatic activations when exposed to certain sulfhydryl reagents, i.e., methylmercuric hydroxide. The activated (10X) methylmercuric derivative has now been crystallized and, in continuing collaboration with the Chemical Department of the University of California, San Diego, the structure of the activated enzyme is now partially known. The structure of a ternary complex of CLDHFR with MTX and the thionicotinamide analog of NADP has also been determined via X-ray analysis which will allow analysis of the role of the carboxamide group in the binding of the pyridine nucleotide to the reductase. The structure of the methylmercuric-activated CLDHFR correlates with conclusions derived from studies on the activation by high concentrations of certain denaturants. Thus, the X-ray data was subjected to distance difference analysis where all of the C-alpha bond distances of the native enzyme are subtracted from the corresponding bond distances in the methylmercuric derivative and the differences are plotted in three dimensions.           </p> <p>             In a continuing collaborative study with the Human Nutrition Research Center, USDA, Beltsville, Maryland, the effects of a controlled intake of various carotenoid-containing foods on the steady-state plasma concentrations of carotenoids was determined. Groups of men were fed daily a relatively large intake of carrots, broccoli, tomato juice or pure beta-carotene for six weeks. Blood was analyzed periodically for seven carotenoid pigments by HPLC.           </p>								

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 DK 15400-15 LCDB												
PERIOD COVERED October 1, 1988 to September 30, 1989														
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Hormones, Lipoprotein Lipase and Lipid Metabolism														
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">P.I.: Robert O. Scow</td> <td style="width: 33%;">Chief, Endocrinology Section</td> <td style="width: 33%;">LCDB:NIDDK</td> </tr> <tr> <td>Others: Hiroshi Masuno</td> <td>Visiting Fellow</td> <td>LCDB:NIDDK</td> </tr> <tr> <td>E. Joan Blanchette-Mackie</td> <td>Research Biologist</td> <td>LCDB:NIDDK</td> </tr> <tr> <td>Albert E. Spaeth</td> <td>Chemist</td> <td>LCDB:NIDDK</td> </tr> </table>			P.I.: Robert O. Scow	Chief, Endocrinology Section	LCDB:NIDDK	Others: Hiroshi Masuno	Visiting Fellow	LCDB:NIDDK	E. Joan Blanchette-Mackie	Research Biologist	LCDB:NIDDK	Albert E. Spaeth	Chemist	LCDB:NIDDK
P.I.: Robert O. Scow	Chief, Endocrinology Section	LCDB:NIDDK												
Others: Hiroshi Masuno	Visiting Fellow	LCDB:NIDDK												
E. Joan Blanchette-Mackie	Research Biologist	LCDB:NIDDK												
Albert E. Spaeth	Chemist	LCDB:NIDDK												
COOPERATING UNITS (if any) Dr. Hiroshi Masuno, Dept of Medical Biochemistry, Ehime University, Ehime, Japan; Dr. Kazuhiro Oka, Medlantic Research Foundation, Washington, DC														
LAB/BRANCH Laboratory of Cellular and Developmental Biology														
SECTION Endocrinology Section														
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892														
TOTAL MAN-YEARS: 1.5	PROFESSIONAL: 1	OTHER: 0.5												
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) Human subjects</td> <td><input type="checkbox"/> (b) Human tissues</td> <td><input checked="" type="checkbox"/> (c) Neither</td> </tr> <tr> <td><input type="checkbox"/> (a1) Minors</td> <td></td> <td></td> </tr> <tr> <td><input type="checkbox"/> (a2) Interviews</td> <td></td> <td></td> </tr> </table>			<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input checked="" type="checkbox"/> (c) Neither	<input type="checkbox"/> (a1) Minors			<input type="checkbox"/> (a2) Interviews					
<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input checked="" type="checkbox"/> (c) Neither												
<input type="checkbox"/> (a1) Minors														
<input type="checkbox"/> (a2) Interviews														
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>Mice born with combined lipase deficiency (<u>cl</u>d/<u>cl</u>d) have very low levels of lipoprotein lipase and hepatic lipase activities, develop extreme hypertriglyceridemia, and die within 3 days. The recessive mutation (<u>cl</u>d) causing this deficiency is located on chromosome 17, whereas structural genes for the lipases are located on chromosomes 9 and 11, respectively.</p> <p>Primary cultures of brown adipocytes derived from tissue of newborn mice were used to study the effect of the <u>cl</u>d mutation on lipoprotein lipase. Cells cultured from brown adipose tissue of <u>cl</u>d/<u>cl</u>d mice replicated, became confluent, and differentiated into brown adipocytes at the same rates as cells of unaffected mice. Unaffected adipocytes synthesized active (dimeric), secretable lipoprotein lipase (Mr=58000) which contained two complex oligosaccharide chains and could be released from cells by heparin. <u>Cl</u>d/<u>cl</u>d adipocytes also synthesized fully glycosylated lipoprotein lipase (Mr=56000), some of which was dimerized, but the lipase contained only high mannose type oligosaccharides, and was inactive and not secretable. Immunolocalization studies showed that the lipase in <u>cl</u>d/<u>cl</u>d adipocytes was retained in endoplasmic reticulum. Unaffected brown adipocytes treated with swainsonine, an inhibitor of mannosidase II in Golgi, synthesized a high mannose type lipoprotein lipase which was both active and secreted, indicating that trimming/processing of high mannose oligosaccharide chains in Golgi may not be necessary for activity or secretion of lipoprotein lipase. Whether the <u>cl</u>d mutation affects primarily processing of oligosaccharides in endoplasmic reticulum, transport of lipase from the reticulum, or some other process, is to be resolved.</p>														

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 15401-17 LCDB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Synthesis and Transport of Lipids and Enzymes in Cells and Tissues

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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Others:	Carmen Mateo	Visiting Fellow	LCDB:NIDDK
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	E. Joan Blanchette-Mackie	Research Biologist	LCDB:NIDDK
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## COOPERATING UNITS (if any)

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## LAB/BRANCH

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## SECTION

Endocrinology Section

## INSTITUTE AND LOCATION

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## TOTAL MAN-YEARS:

3.0

## PROFESSIONAL:

2.0

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Mice born with combined lipase deficiency (cld/cld) have very low levels of lipoprotein, hepatic and lingual lipase activities, have tissues devoid of fat, develop severe hypertriglyceridemia, and die within three days. Recent studies (Z01 DK 15400-15 LCDB) showed that brown adipocytes cultured from cld/cld mice synthesize an inactive, high-mannose form of lipoprotein lipase which is retained in endoplasmic reticulum. The recessive mutation (cld) causing the deficiency is located on chromosome 17, whereas structural genes for the lipases are located on other chromosomes.

The effect of the cld mutation on sensitivity of brown adipocytes to insulin was studied by measuring incorporation of (U-14C) glucose into triacylglycerol in cells cultured from tissue of newborn mice. Cld/cld cells had the same response to insulin during 1-4 hours as unaffected cells, with no response at 0.1 nM and maximal response at 1.0 nM. About 20% of 14C incorporated into triacylglycerol was recovered in the acyl moiety and 80% in the glyceryl moiety. The findings suggest that reception and transmission of the insulin signal, as well as transport of glucose and synthesis of fatty acid and triacylglycerol, are unaffected by the cld mutation.

The effect of the cld mutation on hormone-stimulated lipolysis was also studied in cultured newborn brown adipocytes. Cld/cld brown adipocytes released glycerol from intracellular fat in response to isoproterenol at the same concentrations and at the same rates as unaffected cells. The minimal effect at two hours occurred at 0.5 nM and the maximal at 100 nM. The findings suggest that activation and activity of hormone-sensitive lipase, as well as reception and transmission of the hormone (isoproterenol) signal, are not affected in brown adipocytes by the cld mutation.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 15404-05 LCDB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Ultrastructural Immunocytochemistry of Lipid Metabolism in Cultured Cells and Tissues

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	E. Joan Blanchette-Mackie	Research Biologist	LCDB:NIDDK
Others:	Robert O. Scow	Chief, Endocrinology	
		Section	LCDB:NIDDK
	Hiroshi Masuno	Visiting Fellow	LCDB:NIDDK
	Nancy K. Dwyer	Biologist	LCDB:NIDDK

COOPERATING INSTITUTIONS: Dr. Thomas Olsson, Dept. Physiol. Chem., Univ. of Umea, Sweden; Dr. Carl Alving, Dept. Membrane Biol., Walter Reed Army Inst. of Res., Washington, DC; Dr. Richard E. Pagano, Dept. Embryol., Carnegie Inst. of Washington, Baltimore, MD; Dr. Peter Pentchev, Dev. Metab. Neurol. Br., NINCDS, NIH; and Dr. Howard S. Kruth, Lab. Exptl. Ather., NHLBI, NIH

## LAB/BRANCH

Laboratory of Cellular and Developmental Biology

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Endocrinology Section

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.5

## PROFESSIONAL:

1

## OTHER:

1.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Combined lipase deficiency (cld/cld) in mice is characterized by marked functional deficiencies of both lipoprotein lipase and hepatic lipase. We used immunocytochemistry to locate intracellular lipoprotein lipase in cultured brown adipocytes derived from cld/cld mice and their unaffected littermates. Monensin treatment of normal cells revealed that lipoprotein lipase is associated with the membrane surface of enlarged Golgi vacuoles and smaller vesicles. This finding suggests that lipoprotein lipase transfer from Golgi to intracellular sites of degradation or secretion may occur in association with membranes of Golgi-derived transport vesicles. Cld/cld brown adipocytes synthesize an inactive high-mannose lipoprotein lipase which accumulates in endoplasmic reticulum and is not transferred to Golgi for further modification to a secretable form of the enzyme.

The Golgi complex is the focus of much attention because of its central role in the processing, sorting and targeting of glycolipids and glycoproteins in eukaryotic cells. We have shown that exogenously-derived LDL-cholesterol is translocated from lysosomes to Golgi membranes in normal fibroblasts and those derived from Niemann-Pick Type C patients with an autosomal-recessive neuro-visceral lipid storage disorder. The Golgi is involved in the metabolism of ceramide to sphingomyelin and glucosylceramide in cultured cells. LDL loading of both normal and NP-C fibroblasts enhances ceramide staining of trans Golgi and suggests that cholesterol is the lipid affecting ceramide accumulation in Golgi membranes. Internalization of LDL-cholesterol does not lead to normal cholesterol levels in plasma membrane of sterol-depleted NP-C fibroblasts. Disruptions of the Golgi complex may be responsible for this and other deficiencies in cholesterol utilization in mutant cells.

## DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 15500-29 LCDB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Large-Scale Processing of Biological Material

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Joseph Shiloach  
Others: Jeanne B. Kaufman  
Raphael Fass  
Michelle van de Walle

Research Chemist  
Biol. Laboratory Technician  
Visiting Fellow  
NRC Fellow

LCDB:NIDDK  
LCDB:NIDDK  
LCDB:NIDDK  
LCDB:NIDDK

## COOPERATING UNITS (if any)

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Laboratory of Cellular and Developmental Biology

## SECTION

Office of the Chief

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

3.5

## PROFESSIONAL:

2.0

## OTHER:

1.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

The Pilot Plant (Biotechnology) Unit combines several different types of activities. It is responsible for the large-scale production of bacteria, mammalian cells and biologically active compounds from various sources. Parallel to this activity, it is conducting process development work associated with these preparations in order to be able to execute them efficiently. In addition, the unit is carrying on research work not necessarily associated with a current project, but work that has long-term implications for the unit's performance.

During the last year, the unit carried out 120 different large-scale preparations, including micro-organism growth in volumes from 10 to 300 liters, mammalian cell growth up to 50 liter volumes and processing of various biological materials.

Development work was done in the growth optimization of various recombinant *E. coli* and other micro-organisms, using the fed batch technique and oxygen enriched air system yielding 100 g/l wet weight of biomass. The bacteria were needed for various products, especially bacterial toxins.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 15503-08 LCDB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Regulation of Developmental Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Alan R. Kimmel	Senior Staff Fellow	LCDB:NIDDK
Others:	Charles L. Saxe, III	Senior Staff Fellow	LCDB:NIDDK
	Stephen A. Saxe	Senior Staff Fellow	LCDB:NIDDK

## COOPERATING UNITS (if any)

Dr. Peter Devreotes, The Johns Hopkins Medical School  
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## LAB/BRANCH

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## SECTION

Developmental Biochemistry Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland

## TOTAL MAN-YEARS:

3

## PROFESSIONAL:

3

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- |   |  |   |
|---|--|---|
| <input type="checkbox"/> (a) Human subjects | <input type="checkbox"/> (b) Human tissues | <input checked="" type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors        |  |   |
| <input type="checkbox"/> (a2) Interviews    |  |   |

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A wide variety of cellular functions are regulated by hormonal, sensory or neuronal signals. In many systems the transduction of these extracellular signals occurs through receptor/G-protein linked effector systems. Dictyostelium has proven an excellent system for studying the relationship between transmembrane signalling and differential gene expression. By acting as an extracellular hormone, cAMP exerts multiple effects on the organism through such specific cell surface receptors systems. We have combined molecular, biochemical, cellular and genetic approaches to study the regulation of developmental programs by signal transduction pathways. Cyclic AMP activation of receptors leads to the accumulation of a variety of intracellular second messengers. We have established linkages between these intracellular signals and the regulated expression of specific gene families during development. Data would suggest that each intracellular signal is required to activate a specific protein kinase. Several genes have been isolated which encode cell surface receptors for cAMP. Each receptor has distinct structural characteristics and is expressed with specific temporal and spatial patterns. It will be interesting to establish the relationship between the different receptor forms and the various intracellular effector systems. The isolation of these genes also allows us to study the interaction of receptors with other cellular components. These include cAMP, G-proteins and the specific kinases which are believed to be involved in receptor desensitization. In a parallel series of experiments we have begun a preliminary molecular analysis of hormonal regulation of adipocytes. Our initial studies have centered on the expression of the secreted lipoprotein lipase and the intracellular hormone sensitive lipase. Data indicate that the lymphokine interleukin-6 is capable of repressing the expression of LPL. Since IL-6 is induced by the extracellular factor cachectin, a negative regulator of LPL, these data suggest that IL-6 may mediate the action of cachectin on adipocytes.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 15506-06 LCDB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Control of Gene Expression in Early Mammalian Development

PRINCIPAL INVESTIGATOR (Last other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	Jurrien Dean	Senior Investigator	LCDB:NIDDK
Others:	Margaret Chamberlin	FAES Graduate Student	LCDB:NIDDK
	Li-fang Liang	IRTA Fellow	LCDB:NIDDK
	Sarah Millar	Visiting Fellow	LCDB:NIDDK
	Dwayne Lunsford	Staff Fellow	LCDB:NIDDK
	Anne Baur	Chemist, GS11	LCDB:NIDDK
	Carol Kasten-Sportes	Guest Worker	LCDB:NIDDK
	Eric Lader	IRTA Fellow	LCDB:NIDDK

## COOPERATING UNITS (if any)

Satoru Shimizu, Aichi Cancer Research Institute, Nagoya, Japan; Frank Robey, LCDO:NIDR; Connie Oliver, LM:NIDR; Tom Fuerst, Molecular Vaccines, Inc.; Nancy Jenkins, MGL:NCI, Frederick; Christine Kozak, LMM:NIAD

## LAB/BRANCH

Laboratory of Cellular and Developmental Biology

## SECTION

Developmental Biochemistry Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

7.6

## PROFESSIONAL:

6.6

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
 ☐ (b) Human tissues
 ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

The mouse zona pellucida is an extracellular glycocalyx comprised of three sulfated glycoproteins (ZP1, ZP2 and ZP3) which surrounds growing oocytes, ovulated eggs and dividing embryos. Specific functions have been ascribed to each protein: ZP3 is the sperm receptor and subsequently induces the sperm acrosome reaction; ZP2 acts as a secondary sperm receptor and is biochemically modified in concert with ZP3 to effect the post-fertilization block to polyspermy; and ZP1 appears to cross-link to co-polymers of ZP2 and ZP3. The genes that code for zona pellucida proteins represent a remarkable developmental system which are coordinately expressed uniquely in oocytes during a two-week window of development. Both Zp2 and Zp3 are single copies genes located on mouse chromosome 5 and 7, respectively. Each is transcribed as a poly-adenylated transcript containing short 5' and 3' untranslated regions and have a single open reading frame sufficient to code for a core protein of 80,217 (ZP2) or 46,307 (ZP3) daltons. Although not present in resting oocytes, the zona transcripts accumulate to become very abundant mRNAs in growing oocytes (0.4% and 1%, respectively) before dramatically declining in ovulated eggs. DNA sequences in the 5' flanking region have been identified which appear to have a regulatory role in the oocyte-specific expression of the zona genes. We have undertaken several strategies to learn more about the biological function of the zona pellucida. A 7 amino acid epitope recognized by a contraceptive anti-ZP3 monoclonal antibody has been identified by recombinant DNA techniques. Mice vaccinated with this peptide produce anti-zona antibodies which have a long-term contraceptive effect in the majority of animals. Because the Zp3 gene is conserved from mouse to man, this contraceptive strategy involving allo-immunization may be widely applicable among mammals.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 15508-02 LCDB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chromatin Structure in Regulation of Mammalian Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Ann Dean  
Others: Qihui GongResearch Chemist  
Visiting AssociateLCDB:NIDDK  
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## COOPERATING UNITS (if any)

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Laboratory of Cellular and Developmental Biology

## SECTION

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## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2

## PROFESSIONAL:

2

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

In order to better understand how control of the orderly expression of the human embryonic, fetal and adult globins is mediated during development, we are studying the changes in chromatin structure that these genes undergo when they are activated. We have used the electrophoretic mobility shift assay to detect interactions between putative trans-acting regulatory factors present in the nuclei of cells actively transcribing globin genes and cis-acting sequences flanking these genes. We detected strong binding sites in the 3' flanking regions of the epsilon-, gamma- and beta-globin genes for an erythroid specific protein in K562 cell nuclear extracts. Competition experiments showed that the same protein interacted with all these sites. Gel mobility shift assays, as well as DNase I footprinting, indicated that this protein is likely to be the human analogue of EryF1, a chicken globin enhancer binding protein. The gamma- and beta-globin binding sites for the protein corresponded to regions of DNA which had been shown to possess enhancer activity for their respective genes either *in vitro*, or *in vivo*. To investigate the functional significance of the binding sites for this protein in regions of DNA 3' to the epsilon-globin gene, transient assays in K562 cells were employed in which enhancement of transcription from the epsilon-globin promoter was monitored via a chloramphenicol acetyltransferase reporter gene. The epsilon-globin promoter is enhancer responsive to this assay. A direct assessment of the functional involvement of the erythroid specific protein in enhancement of transcription can be made by inserting in the expression vector enhancer fragments in which the *in vitro* protein binding sites have been mutated using the polymerase chain reaction.

## ANNUAL REPORT OF THE LABORATORY OF BIOCHEMISTRY AND METABOLISM

### NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The Laboratory conducts research in several apparently disparate areas that include morphogenesis, differentiation, endocytosis, endocrinology, membrane transport, detoxication, and the physical and chemical behavior of proteins and nucleic acids. It does so by applying a broad array of different approaches. Resolution is being attempted by methods that stem from enzymology, carbohydrate chemistry, cell biology, biophysics and molecular biology. Although seemingly diverse, there is a common element to each of the subjects summarized here that is appropriate to the Laboratories' designation: biochemical, metabolic and physical approaches are being brought to bear on major problems encompassed by the Institute's charge. It is the close proximity of experienced investigators from diverse scientific disciplines, able to discuss their individual problems with each other, that provide synergistic effects for the resolution of the questions under investigation.

#### I. ENZYMES: FUNCTIONAL AND ABNORMAL

Several groups are active in this broadly designated area which covers the discovery of the several lesions in the gene for an enzyme whose absence leads to Tay-Sachs disease; the lesion in an enzyme that leads to sialuria; the means by which enzymes play a role in morphogenesis; and the enzymatic mechanism by which aspirin is metabolized.

##### The genetic lesions of Tay-Sachs disease

Tay-Sachs disease is an autosomal recessive disorder caused by mutations in the  $\alpha$ -chain polypeptide of the A form of  $\beta$ -hexosaminidase, a lysosomal enzyme composed of two chains. Such lesions result in a spectrum of disease ranging from severe to mild. The Ashkenazi Jewish population is enriched for carriers of a fatal form of Tay-Sachs disease. It had been presumed that Tay-Sachs patients from this ethnic isolate harbored the same  $\alpha$ -chain mutation. This notion has now been disproved by identification of a splice junction defect in the  $\alpha$ -chain of an Ashkenazi patient. Only 30% of patients had this lesion. In looking for the other 70% of the gene population,  $\alpha$ -chain genomic clones were obtained from other Ashkenazi Jewish patients who did not have the splice junction defect. By sequence analysis of the promoter region, exon and splice junction regions, and the polyadenylation signal area of the mutant gene, a 4-base pair insertion was found in exon 11. This mutation introduces a premature termination signal. A dot blot assay has been developed, utilizing the polymerase chain reaction to amplify the region of exon 11 encompassing the insertion, to screen patients and carriers for the insertion mutation. The lesion was found in about 70% of the carriers tested, thereby distinguishing it as the major defect underlying Tay-Sachs disease in the Ashkenazi Jewish population.

In addition, the DNA of the Ashkenazi Jewish patient used to isolate the  $\alpha$ -chain clone bearing the insertion mutation was found not to be homozygous for this mutation. These results indicated that a third mutation is probably present at very low frequency in the Ashkenazi population. A lambda clone of this allele has been isolated and present work is focused on analyzing and characterizing the third defect.

### The role of the carbohydrate moiety of glycoproteins in cellular activity

Studies on the mechanism of ligand induced modulation of the asialoglycoprotein receptor on hepatoblastoma cells, Hep G2, have shown the resultant down regulation to be correlated with a concurrent loss of cell surface sialic acid. Evidence has now been adduced to establish a causal relation between the metabolism of saturating levels of ligand, such as asialoorosomucoid or N-acetylgalactosamine, and a defect in the biosynthesis of sialic acid. Examination of the enzymatic steps leading from the first committed compound, N-acetylmannosamine, to the end product, sialic acid, were unaffected. Current pulse chase experiments are in progress to determine the nature and locus of the metabolic block.

Sialuria is a rare inborn error of metabolism which is characterized by extraordinarily high levels of free sialic acid in the urine. Utilizing fibroblasts from two patients suffering from this disease, the basic biochemical defect has been identified and shown to be different from that operative in related diseases including sialidosis, Salla disease and infantile sialic storage disease. In normal cells, the production of sialic acid is controlled by a sensitive feedback mechanism whereby CMP-sialic regulates the activity of the rate controlling enzyme in sialic acid biosynthesis. In sialuric patients, sensitivity to this feedback mechanism is missing with resultant overproduction of sialic acid and the accompanying pathological state.

### Polysaccharides in morphogenesis

A model system for morphogenesis is the growth of a bud scar in yeast, that is made of chitin, at the site of division of mother and progeny. A series of enzymes that include chitin synthetase participate but there are two such enzymes. Chitin synthetase 2 is essential for septum formation and cell division in yeast. Chitin synthetase 1 replenishes chitin lost in certain emergency situations, but cannot substitute for chitin synthetase 2. Determination of the sequence of the CHS2 gene and comparison with that of CHS1 revealed that the two enzymes share an area of homology extending over about two-thirds of the sequence on the carboxyl terminal side. On the other hand, the amino terminal portions shows no similarities and may determine the different physiological functions of the two synthetases.

A protocol has been developed for partial purification of the GTP-binding component of  $\beta(1-3)$ glucan synthetase, the enzyme that catalyzes the formation of the principal structural polysaccharide of the yeast cell wall.

### Enzymatic basis of detoxication

As part of a study of the enzymatic means by which foreign compounds are removed from the body, attention was given to a very common drug. Although aspirin (acetylsalicylic acid) is known to be hydrolyzed to salicylic acid as the initial step in its metabolism, little is known of enzyme mechanism for a drug of such importance. Preliminary studies of aspirin hydrolysis have shown that about half of the cell's hydrolytic activity is localized in membranes and the other half is in a soluble form.

Two cytosolic enzymes were found to have very high affinity for aspirin. These two have now been purified to electrophoretic homogeneity and appear to be isoenzymes in that they have entirely similar substrate specificity and react to an antibody raised to one of them. Specificity of the reaction is directed to aromatic esters of short-chain acids with acetates hydrolyzed faster than butyrates. Both enzymes have the characteristics of serine-esterases. Kinetic studies with a number of substituted phenylacetates suggest that the limiting step in enzyme catalyzed hydrolysis is the release of acetate from the enzyme.

## II. PROTEIN SORTING AND TRANSPORT FUNCTIONS

Central to modern biology is the nature of the mechanism for the movement of macromolecules, glycoproteins in particular, not only into and out of the cell but also into specific organelles. Plural mechanisms are being sought from the viewpoint of the disciplines of somatic cell genetics, molecular biology, carbohydrate chemistry, endocrinology and biochemistry. The implications of the work extend from cell biology to applications in thyroid pathobiology and approaches to the AIDS virus.

### Endocytosis, secretion and compartmentalization in mutant CHO cells

One approach to dissecting the processes of endocytosis, glycoprotein biosynthesis and sorting is through isolation and analysis of mutants. Two classes of endocytosis mutants in CHO cells, End 1 and End 2, appeared to be defective in endosomal but not lysosomal acidification, based on assays of ATP-dependent acidification of organelles recovered from Percoll gradients. However, recent examination of living cells using the ACAS 470 cell sorter, indicated that lysosomal acidification also was decreased in the mutants. Pursuing this discrepancy, centrifugation through Percoll was found to significantly reduce ATP-dependent acidification of lysosomes from normal cells. Using a modified labeling procedure whereby a pH reporter is compartmentalized only in lysosomes, was found to assess lysosomal acidification without fractionation: ATP-dependent lysosomal acidification in End 1 and 2 mutants decreases in parallel with endocytic activity.

Evidence for regulation of both the biosynthesis and compartmentalization of the Man6P/IGFII receptor was also obtained. On return of a temperature-sensitive End 1 mutant from the non-permissive to the permissive temperature, rapid degradation of pre-existing receptor and rapid disappearance of receptor from the cell surface takes place. However, the total number of Man6P/IGFII receptors remains constant due to a compensating increases in biosynthesis. By a yet to be defined mechanism, these new receptors mix with the endocytic pathway at one-tenth of the normal rate.

### The role of the nuclear envelope in intracellular protein sorting

The entry of proteins into the cell nucleus plays a central role in the regulation of cell growth and development. A short segment from the coding sequence of the SV40 Large T antigen is required for nuclear transport of T antigen to the nucleus. When chemically coupled to the large fluorescent protein B-phycoerythrin, synthetic peptides corresponding to this sequence specifically target the protein conjugate to the nucleus. Mutations in the sequence prevent nuclear transport. An in vitro binding and import assay was

developed using isolated rat liver nuclei and labelled peptide conjugates. Conjugates having the proper sequence bind to nuclei with a  $K_d=100\text{nM}$ ; only conjugates containing the localization sequence bind with high affinity. Monoclonal antibodies have been generated which bind specifically to the SV40 localization sequence and block nuclear transport of T antigen. The nuclear pore complex mediates transport across the nuclear membrane. The laboratory has demonstrated that proteins bearing cytoplasmically oriented, O-linked GlcNAc are components of the nuclear pore complex. Monoclonal antibodies and the lectin wheat germ agglutinin bind to these proteins and block import into the nucleus and the export of RNA from the nucleus to the cytoplasm. These findings suggest that the glycoproteins may be involved in the assembly or functioning of the nuclear pore. Sequence information from the major nuclear pore protein (p62) was obtained and this protein has been molecularly cloned. Full length cDNA and genomic clones have been isolated. The nuclear pore protein has two domains; one myosin-like and one collagen-like. Anti-peptide antibodies were generated to various regions of the p62 coding sequence. By transfecting an expression construct encoding p62 it has been possible to overexpress this protein in cultured cells. The gene is highly conserved from yeast to man. Efforts are underway to identify the yeast homologue of p62 so that yeast genetics and molecular techniques can be used to determine the normal function of p62.

#### Electrochemical ion gradients as a mechanism of cellular message transmission

The project involves the study of iodine metabolism in the thyroid. Prior studies used thyroid cells in culture to characterize properties of iodide transport, and described an iodoglycolipid that was an intermediate in iodine metabolism. (a) Studies over the last year focused on defects in iodine metabolism that lead to a decrease in thyroid hormone formation and enlargement of the thyroid (goiter). Three types of goiter were studied: i) goiter due to an iodine deficient diet, ii) goiter with a congenital defect in thyroglobulin synthesis with dyshormonogenesis, and (iii) defect in sialiation of thyroglobulin that could not be secreted into the thyroid follicle. Biochemical studies of thyroglobulin prepared from these goitrous thyroids revealed alternative pathways of thyroid hormone formation in the thyroid. Studies also involved chemical modifications of thyroglobulin. These studies use reagents that react selectively with lysyl residues. Thyroglobulin for these studies were the same as described above as well as from normal human and bovine thyroids. Treatment with these reagents releases low molecular weight peptides. Release of iodopeptides also occurs spontaneously. Since most thyroid hormones are contained in this area of the thyroglobulin molecule, the interaction of the peptide with thyroglobulin may have an important role in the synthesis and release of thyroid hormones from thyroglobulin. (b) Studies characterizing the iodide transport protein in the thyroid have continued, and take advantage of the similarity with the anion transport protein known as band 3 from erythrocytes. In prior studies, agents interacting specifically with band 3 were shown to also affect the transport of iodide by the thyroid. Monoclonal antibodies raised against band 3, also block iodide transport. Now, several clones were isolated using a RNA expression library from thyroid cells, and hybridized with a cDNA probe to murine band 3. Studies have been initiated using these clones to express iodide transport activity in *Xenopus* oocytes.

### Cell regulation by pharmacodynamic and autoimmune agents acting on cell membranes

The laboratory is evaluating structure-function relationships between glycoprotein hormones (thyrotropin), autoantibodies, oncogenes,  $\alpha$  1-adrenergic agents, insulin, insulin-like growth factors (I and II), bacterial toxins (cholera and pertussis, for example), interferon, and interleukins - alone and in combination. It is studying the mechanisms, both transcriptional and posttranscriptional, by which they interact with and transmit their message through the cell membrane to affect cell function and pathology. Studies using monoclonal antibodies and the idiotype antiidiotype theory explore the structure of the receptors for these ligands and the importance of these relationships to the expression of thyroid hyperfunction in Graves' disease; to organ-specific autoimmunity (Graves' disease, Hashimoto's disease, lupus, diabetes); to fluid losses in intestinal diarrheic states; to thyroid storm and the sympathetic overactivity syndrome of tetanus; to the ability of hormones to modulate the oncogenic state; and to the mechanism by which toxins subvert normal mechanisms to impose their pathological effects. Studies continue to evaluate the role of different signal transduction mechanisms - cAMP, Ca/phosphoinositide and arachidonate - for growth and differentiation, thyroglobulin biosynthesis, lipid/cholesterol biosynthesis, thyroglobulin biodegradation to T3 and T4, and the transport of T3, T4, monoiodotyrosine, diiodotyrosine, or other amino acids from the lysosome. The role of phosphate and carbohydrate regions in the thyroglobulin structure and in post-translational processing is being studied. Studies explore lipid regulation of receptor expression, with special emphasis on thyroid cell growth and development, lipid metabolism, LDL receptor expression, and cholesterol biosynthesis. Studies to clone the TSH receptor and other autoantigens important to developing or expressing autoimmune Graves' and exophthalmos, as well as to define their structure and regulatory control at a gene level, are in progress as are studies to define the mechanisms by which TSH, insulin, IGF-I and other ligands regulate gene expression.

### The role of intracellular traffic in HIV infection

CD4, the presumed T-cell receptor for the human immunodeficiency virus, depends upon glycosylation for proper surface expression. Tunicamycin, a potent inhibitor of glycosylation blocks expression of CD4 at the cell surface under conditions where alternate surface receptors are unaffected. Initial studies employing acute lymphoblastic leukemic cells have been extended by the successful transfection of a plasmid containing the cDNA for CD4 into mutant and wild type Chinese hamster ovary cells. Subsequent cotransfection with another plasmid containing the multiple drug resistant gene, has permitted the isolation of stable clones expressing large amounts of CD4. Characterization of these clones suggests that CD4 contains biantennary unsialylated complex-type oligosaccharides. The data also suggest that the inability of CD4 to reach the cell surface when it lacks carbohydrate is due to the selective degradation of unglycosylated CD4.

A targeting sequence has been identified which is sufficient to allow protein molecules as large as  $0.5 \times 10^6$  to pass through the nuclear envelope. This has been assessed in our laboratory by chemically coupling peptides having the nuclear localization sequence to a highly fluorescent cytoplasmic protein. The sequence Pro-Lys-Lys-Lys-Arg-Lys-Val-Cys is sufficient to target these

conjugates to the nucleus. The tat and art gene products of HIV have sequences very similar to this targeting domain. In studies, we have begun to examine the structure of the nuclear pore complex, across which the many nucleocytoplasmic exchange processes must occur. The structure of the pore complex is explored using recombinant DNA techniques. The major nuclear pore glycoprotein np62 has been cloned, sequenced and expressed in cultured cells. The gene encoding this protein has been isolated and found to be 2.95 kb in length and devoid of introns. The availability of the primary sequence of this protein has allowed the preparation of anti-peptide antibodies which react with p62. These antibodies should prove useful for probing the function of the pore complex in the HIV life cycle.

### III. REGULATION OF DNA BY PROTEINS

The interaction of specific proteins with DNA is probably the major regulatory guide of growth and differentiation. These effects have been demonstrated for a specific physiological function, lactation, and for viral behaviour.

#### Tissue specific and hormone regulated gene expression

The molecular basis of mammary specific and hormone regulated gene expression is being studied through analysis of cis-acting regulatory elements of the mouse whey acidic protein (WAP) gene using in vitro systems and transgenic animals. It was shown that the promoter of the mouse whey acidic protein gene contains elements conferring mammary specific gene expression in transgenic animals, but in contrast to the endogenous gene, is not subject to control by insulin, hydrocortisone and prolactin. Using in vitro protein-DNA binding assays, the WAP and  $\alpha$ -lactalbumin promoters share sequence motifs are recognized by nuclear proteins, suggesting that they play some role in the regulation process. This was confirmed for the WAP gene by using an in vitro transcription system. In continuation of work with the mammary gland as a bioreactor or production of valuable pharmaceuticals, the human CD4 receptor in the milk of transgenic animals was expressed.

The structure and function of the control region of the human cytomegalovirus immediate early 1 gene has been studied using in vitro systems which partially mimic the regulation observed in vivo. After having analyzed the promoter/enhancer region, the modulatory region was studied. This region, which is located 5' of the enhancer, can act in a cell-specific manner as a transcriptional activator or repressor and is recognized by nuclear proteins.

#### Cell specific activity of elements within the HIV-LTR

One clinical suggestion, that HIV may be activated after exposure to steroid hormones, is the observation that HIV p24 antigenemia is more frequently found in pregnant rather than non-pregnant women and that this antigenemia disappears after parturition. Transcription from the HIV-LTR was studied, therefore, in the presence or absence of hormonal stimuli in tissue culture cells and transgenic animals. In the presence of dexamethasone, transcription from the HIV-LTR in tissue culture cells was elevated at least 4-fold. Moreover, expression from the HIV-LTR was elevated in transgenic elements during pregnancy, suggesting that steroid hormones may contribute to HIV expression during pregnancy.

The DNA sequences which mediate the steroid hormone action on the HIV-LTR are currently being identified and their interaction with known transcription elements in the HIV-LTR are being analyzed.

#### IV. PHYSICAL ASPECTS OF MEMBRANES, TRANSPORT AND PROTEINS

The more physical attributes of proteins, lipids, and DNA, as well as their interaction in membranes and function in transport, is the concern of several groups in the Laboratory.

##### Physics of ionic channels and other proteins with aqueous cavities

The laboratory has been studying membrane transport proteins with regard to the physical factors that govern voltage gating and lipid protein interactions. Beginning with the application of osmotic stress to determine the amount of water that enters an opening channel, finding that this amount is large enough to imply significant changes in protein arrangement, recognizing that such changes will in turn lead to different modes of protein packing in lipids, present work is in varying the host lipids into which purified membrane proteins are incorporated during functional reconstitution. Preliminary results suggest that lipids affect not only voltage gating properties but also the ability of proteins to move into lipid bilayers.

A practical technique has been developed for incorporating a single protein into lipid vesicles that can be fused into planar bilayers for transport measurements. Differences in vesicular sedimentation often suffice to allow separation of vesicles containing different numbers or kinds of proteins.

It has also been shown that vesicles created from rough endoplasmic reticulum contain proteins that conduct electrical current when introduced into planar bilayers by vesicular fusion. This finding gives important support that protein translocation is associated with transport through pores.

##### Direct measurement of forces between membranes or macromolecules

By directly measuring the forces between large molecules or between membranes we are learning finally how these interactions allow the formation of molecular assemblies. Beginning with the discovery that hydration or solvation forces are the most important interactions encountered during the last few nanometers approaching contact, systematically investigations are being conducted on the molecular features that govern surface hydration. Hydration forces are very sensitive to relatively small changes in composition of lipid polar groups or of small molecules bound to the macromolecular surface.

Following the measurement of the change in molecular disorder accompanying changes in molecular interaction with separation, it has been possible to derive a model of molecular motion in the force field of neighboring molecules. There has finally been a connection established between molecular force and motion.

On the theoretical side also, analysis of the fluctuations of ionic charge suggested that the resulting charge fluctuation forces, analogous to van der Waals forces, create strong attractive forces that explain the puzzling "long range hydrophobic interaction" between non-polar surfaces. Earlier ideas about this interaction had to assume improbable perturbation of water solvent hundreds of Angstroms from the surface.

In the study of lipid forces, success has been attained in measuring the energies of non-lamellar lipid phases. It appears now that many cell membrane lipids that exist in lamellar form are actually under a very great level of bending strain having been forced from the curved conformation that they would otherwise prefer. Recognition of this strain has made clear that the incorporation of membrane proteins involves significant energies of protein lipid interaction of bending.

Most recently, NMR is being used to detect the influence of membrane lipids on boundary water. It is becoming possible to make a connection between these resonance measurements and the directly measured forces between lipid bilayers.

#### Cell-cell fusion due to influenza hemagglutinin

Studies are continuing on the infection process by which enveloped viruses, such as influenza, rabies, herpes and HIV, inject nucleic acids into the cytoplasm. This invasion is initiated via membrane fusion: the bound virus fuses to the plasma membrane or internalized membrane after receptor-mediated endocytosis. Study of the fusion process utilizes directly a NIH3T3 fibroblast which expresses the influenza HA fusion protein in its surface. Thus the fibroblast surface has molecular features of the influenza virus, and will bind human red cells. Upon a change in external pH, the two cells fuse. Fusion is measured in three physically different ways: lipid exchange, cytoplasm exchange, and cell surface area change. These techniques indicate that influenza hemagglutinin-induced fusion rapidly establish both bilayer continuity and exchange of cytoplasmic contents. By using low light level video microscopy and image enhancement techniques, the spatial relocation of both dyes is followed in single cells during the fusion process. This study revealed the following new observations and insights into HA-induced cell fusion: i) Continuous monitoring of fluorescence changes using two different criteria for fusion showed that fusion is rapid, the maximal extent is reached within minutes; (ii) The correspondence of the kinetics of the aqueous and lipid probes indicates that the cytoplasmic connections form as rapidly as the outer bilayers mix and there is no long-lived "partial-fusion" intermediate; (iii) Movement of fluorophores between effector and target is restricted during the initial events in fusion, consistent with the opening of small junctional pore(s); (iv) HA-induced leakage of a small molecule from the target occurred concomitant with fusion.

#### Histamine release from beige mouse mast cells

Mast cells of the beige mouse contain large intracellular secretory granules (approximately 4 microns in diameter) whose membranes fuse with the plasma membrane in a process called exocytosis. During membrane fusion an exocytotic pore forms which connects the granule interior with the extracellular medium. Through the exocytotic pore the granule contents are released extracellularly and are free to diffuse to target cells.

Electrophysiological and light microscopic data are now being used in an investigation of the structure of this exocytotic pore during secretion in mast cells from Beige mice. The time course for the widening of the pore is highly variable: it can widen quickly or slowly and can fluctuate between dilated and contracted states of variable conductance (flickering). Initial pore sizes are broadly distributed indicating that this pore is different from

traditional membrane channels which have a relatively fixed conductance. The frequency histogram for occurrence of pores of given conductance is broad with a primary peak between 1 and 4 nS, indicating that pore size does not increase in quantal steps. A secondary maximum occurs at about 30 nS. Fast-frozen, freeze-fracture replicas of rat mast cells have been searched and pores identified with small lumens. The 30 nS pores may represent the smallest pores seen in transmission electron microscopy. A model describing fusion on the molecular level which can account for the variable pore sizes, flickering, and known volumes of activation is described.

In a second project, isolated matrices of the giant secretory vesicles of Beige mouse mast cells were examined to determine the effects of the ionic composition of the bathing solution on their size. In general, multivalent cations condense the matrix relative to univalents.

#### Thermodynamic and kinetic studies of protein structure and enzymic mechanisms

This laboratory is engaged in studies on protein structure and the mechanism by which a protein molecule, which is synthesized as a random coil, can fold into a specific secondary and tertiary structure, without any external help. The main subject of research is swine pepsinogen, a monomeric protein of molecular weight=39,630, which is stable at pH's between 6 and 8.5. Below pH 6 pepsinogen activates itself by proteolytic loss of it's first 44 amino acids, to produce an enzymatically active protein, pepsin. Pepsin is stable only at pH's below 6. Both proteins are unfolded by exposure to high pH, temperature or concentrations of denaturants, such as urea. After such unfolding, pepsinogen can refold to its normal structure, when returned to native conditions, whereas pepsin cannot. I am interested in the mechanism of this refolding reaction and on the influence of the change in sequence on the behaviour of the two proteins. Using techniques such as ultra-violet, circular dichroic and fluorescence spectroscopies, together with chemical modification and peptide chemistry, the structures of the native and unfolded species have been characterized. Using rapid kinetic techniques, such as stopped-flow and T-jump, intermediate, partly folded forms have been detected in the folding reaction, their structures have been partially determined and the nature of the chemical reactions which separate them from the native and unfolded forms investigated.

#### Structure and physical properties of DNA and DNA-protein complexes

Extending previous results for myosin II monomer and dimer structure, bipolar myosin filaments with electric birefringence are being examined. The orientation of the complex is entirely due to a field induced perturbation of the internal structure. One is able, therefore, to probe directly the dynamics of hinge (HMM-IMM junction) region motion. The precise role of hinge flexibility on force generation during the actin activated ATPase cycle of myosin is as yet unclear. It is now possible, however, to quantitatively relate hinge motion with ATPase activity. The limiting structure observed is consistent with a bipolar filament composed of 16 monomers. Hinge motion is best described by a bending spring rather than a free joint. Phosphorylation of the C-terminal control region dramatically changes the flexibility of the hinge region, presumably through the close proximity of hinge and control region on neighboring myosins. That phosphorylation also modulates ATPase activity strongly suggests that hinge motion is involved in force generation.

Within the past several years, it has become clear that DNA can adopt a variety of alternate B-form structures depending on sequence and ionic conditions. These structures may play a role in the organization of DNA in protein complexes, e.g. in nucleosome positioning. The 5s RNA gene from sea urchin and *Xenopus* does form a uniquely positioned nucleosome. This DNA fragment also shows unusual flexibility with counterions that bind into grooves rather than to the sugar-phosphate backbone. This effect is not general for all DNA sequences and is due to either static bending or anisotropic flexing. Oligopurine stretches of length 5 bp or greater appear to be the affected sequences. The distribution of oligopurine sequences in these DNA fragments and anisotropic bending correlate well with nucleosome positioning.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 17001-23 LEM
PERIOD COVERED <u>October 1, 1988</u> to <u>September 30, 1989</u>		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <u>The Role of the Carbohydrate Moiety of Glycoproteins in Cellular Activity</u>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:  Others:	G. Ashwell  R. Koenig P. Weiss	Institute Scholar  Visiting Fellow Visiting Associate  LEM, NIDDK  LEM, NIDDK LEM, NIDDK
COOPERATING UNITS (if any) NICHD, NIH (Dr. William Gahl)		
LAB/BRANCH <u>Laboratory of Biochemistry and Metabolism</u>		
SECTION <u>Section on Enzymes and Cellular Biochemistry</u>		
INSTITUTE AND LOCATION <u>NIDDK, NIH, Bethesda, Maryland 20892</u>		
TOTAL MAN-YEARS:  <div style="text-align: center;">3</div>	PROFESSIONAL:  <div style="text-align: center;">3</div>	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>           Studies on the mechanism of ligand induced modulation of the asialoglycoprotein receptor on hepatoblastoma cells, Hep G2, have shown the resultant down regulation to be correlated with a concurrent loss of cell surface sialic acid. Evidence has now been adduced to establish a causal relation between the metabolism of saturating levels of ligand, such as asialoorosomucoid or N-acetylgalactosamine, and a defect in the biosynthesis of sialic acid. Examination of the enzymatic steps leading from the first committed compound, N-acetylmannosamine, to the end product, sialic acid, were unaffected. Current pulse chase experiments are in progress to determine the nature and locus of the metabolic block.         </p> <p>           Sialuria is a rare inborn error of metabolism which is characterized by extraordinarily high levels of free sialic acid in the urine. Utilizing fibroblasts from two patients suffering from this disease, the basic biochemical defect has been identified and shown to be different from that operative in related diseases including sialidosis, Salla disease and infantile sialic storage disease. In normal cells, the production of sialic acid is controlled by a sensitive feedback mechanism whereby CMP-sialic regulates the activity of the rate controlling enzyme in sialic acid biosynthesis. In sialuric patients, sensitivity to this feedback mechanism is missing with resultant overproduction of sialic acid and the accompanying pathological state.         </p>		

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 17002-19 LEM

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Enzymatic Basis of Detoxication

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: W.B. Jakoby Chief, LEM LEM, NIDDK

Others: Y-S. Yang Visiting Fellow LEM, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Biochemistry and Metabolism

## SECTION

Section on Enzymes and Cellular Biochemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2

## PROFESSIONAL:

1.5

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Although aspirin (acetylsalicylic acid) is known to be hydrolyzed to salicylic acid as the initial step in its metabolism, little is known of enzyme mechanism for such an important drug. Preliminary studies of aspirin hydrolysis have shown that about half of the cells hydrolytic activity is localized in membrane components and the other half is in a soluble form.

Two cytosolic enzymes were found to have very high affinity for aspirin. These two have now been purified to electrophoretic homogeneity and appear to be isoenzymes in that they have entirely similar substrate specificity and react to an antibody raised to one of them. Specificity of the reaction is directed to aromatic esters of short-chain acids with acetates hydrolyzed faster than butyrates. Both enzymes have the characteristics of serine-esterases. Kinetic studies with a number of substituted phenylacetates suggest that the limiting step in enzyme catalyzed hydrolysis is the release of acetate from the enzyme.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 17003-22 LEM

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Polysaccharides in Morphogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	E. Cabib	Senior Research Chemist	LEM, NIDDK
Others:	H.-M. Park	Visiting Fellow	LEM, NIDDK
	J.A. Shaw	IRTA	LEM, NIDDK
	S.J. Silverman	Expert	LEM, NIDDK

COOPERATING UNITS (if any)

Laboratory of Cell Biology, NHLBI, NIH (B. Bowers)

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

3.7

PROFESSIONAL:

3.7

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

Chitin synthetase 2 is essential for septum formation and cell division in yeast. Chitin synthetase 1 replenishes chitin lost in certain emergency situations, but cannot substitute for chitin synthetase 2. Determination of the sequence of the CHS2 gene and comparison with that of CHS1 revealed that the two enzymes share an area of homology extending over about two-thirds of the sequence on the carboxyl terminal side. On the other hand, the amino terminal portions shows no similarities and may determine the different physiological functions of the two synthetases.

A protocol has been developed for partial purification of the GTP-binding component of Beta(1-3)glucan synthetase, the enzyme that catalyzes the formation of the principal structural polysaccharide of the yeast cell wall.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 17004-21 LEM

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Thermodynamic and Kinetic Studies of Protein Structure and Enzymic Mechanisms

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Peter McPhie Research Chemist LEM, NIDDK

COOPERATING UNITS (if any)

IMB, NCI (Jane Cheng); Georgetown University, Dept. of Biochemistry (Preston Hensley, Assoc. Prof.); LPD, NIAID (Russell Howard); DCRT (Richard Shrager)

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1

PROFESSIONAL:

1

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

This laboratory is engaged in studies on protein structure and the mechanism by which a protein molecule, which is synthesized as a random coil, can fold into a specific secondary and tertiary structure, without any external help. The main subject of research is swine pepsinogen, a monomeric protein of molecular weight=39,630, which is stable at pH's between 6 and 8.5. Below pH 6 pepsinogen activates itself by proteolytic loss of it's first 44 amino acids, to produce an enzymatically active protein, pepsin. Pepsin is stable only at pH's below 6. Both proteins are unfolded by exposure to high pH, temperature or concentrations of denaturants, such as urea. After such unfolding, pepsinogen can refold to its normal structure, when returned to native conditions, whereas pepsin cannot. I am interested in the mechanism of this refolding reaction and on the influence of the change in sequence on the behaviour of the two proteins. Using techniques such as ultra-violet, circular dichroic and fluorescence spectroscopies, together with chemical modification and peptide chemistry, the structures of the native and unfolded species have been characterized. Using rapid kinetic techniques, such as stopped-flow and T-jump, intermediate, partly folded forms have been detected in the folding reaction, their structures have been partially determined and the nature of the chemical reactions which separate them from the native and unfolded forms investigated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 17008-06 LEM

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Role of the Nuclear Envelope in Intracellular Protein Sorting

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J.A. Hanover	Senior Staff Fellow	LEM, NIDDK
Others:	M. D'Onofrio	Visiting Fellow	LEM, NIDDK
	T. Olson	Special Volunteer	LEM, NIDDK
	M.K. Park	Visiting Fellow	LEM, NIDDK
	C. Starr	IRTA	LEM, NIDDK
	B. Wolff	Visiting Associate	LEM, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

4.2

PROFESSIONAL:

4.2

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The entry of proteins into the cell nucleus plays a central role in the regulation of cell growth and development. A short segment from the coding sequence of the SV40 Large T antigen is required for nuclear transport of T antigen to the nucleus. When chemically coupled to the large fluorescent protein B-phycoerythrin, synthetic peptides corresponding to this sequence specifically target the protein conjugate to the nucleus. Mutations in the sequence prevent nuclear transport. We have developed an *in vitro* binding and import assay using isolated rat liver nuclei and labelled peptide conjugates. Conjugates having the proper sequence bind to nuclei with a  $K_d=100\text{nM}$ ; only conjugates containing the localization sequence bind with high affinity. Monoclonal antibodies have been generated which bind specifically to the SV40 localization sequence and block nuclear transport of T antigen. The nuclear pore complex mediates transport across the nuclear membrane. The laboratory has demonstrated that proteins bearing cytoplasmically oriented, O-linked GlcNAc are components of the nuclear pore complex. Monoclonal antibodies and the lectin wheat germ agglutinin bind to these proteins and block import into the nucleus and the export of RNA from the nucleus to the cytoplasm. These findings suggest that these glycoproteins may be involved in the assembly or functioning of the nuclear pore. Sequence information from the major nuclear pore protein (p62) was obtained and this protein has been molecularly cloned. Full length cDNA and genomic clones have been isolated. The nuclear pore protein has two domains; one myosin-like and one collagen-like. Anti-peptide antibodies were generated to various regions of the p62 coding sequence. By transfecting an expression construct encoding p62 it has been possible to overexpress this protein in cultured cells. The gene encoding p62 is devoid of introns and has two transcriptional start sites. The gene is highly conserved from yeast to man. Efforts are underway to identify the yeast homologue of p62 so that yeast genetics and molecular techniques can be used to determine the normal function of p62.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 17009-04 LEM

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Tissue Specific and Hormone Regulated Gene Expression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: L. Hennighausen Visiting Associate LEM, NIDDK

Others: T.G. Burdon Special Volunteer LEM, NIDDK  
P.A. Furth Special Volunteer LEM, NIDDK  
R.A. McKnight IRTA LEM, NIDDK  
H.H. Niller Special Volunteer LEM, NIDDK  
C. Pittius Special Volunteer LEM, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

4.2

PROFESSIONAL:

4.2

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The molecular basis of mammary specific and hormone regulated gene expression is being studied through analysis of cis-acting regulatory elements of the mouse whey acidic protein (WAP) gene using in vitro systems and transgenic animals. It was shown that the promoter of the mouse whey acidic protein (WAP) gene contains elements conferring mammary specific gene expression in transgenic animals (1) but in contrast to the endogenous gene is not subject to control by insulin, hydrocortisone and prolactin (2). Using in vitro protein-DNA binding assays it was shown that the WAP and alpha-lactalbumin promoters share sequence motifs which are recognized by nuclear proteins (3,4) suggesting that they play some role in the regulation of these genes. This was confirmed for the WAP gene using an in vitro transcription system (5). In continuation of using the mammary gland as a bioreactor to produce valuable pharmaceuticals, we expressed the human CD4 receptor in the milk of transgenic animals (6).

The structure and function of the control region of the human cytomegalovirus (HCMV) immediate early 1 (IE1) gene has been studied using in vitro systems which partially mimic the regulation observed in vivo. After having analyzed the promoter/enhancer region, the modulatory region was studied. This region, which is located 5' of the enhancer, can act in a cell-specific manner as a transcriptional activator or repressor and is recognized by nuclear proteins (7,8).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 17024-06 LEM

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Genetic Lesions of Tay-Sachs Disease

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: R. Myerowitz Research Chemist LEM, NIDDK

Others: S. Shore Special Volunteer LEM, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Enzymes and Cellular Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.75

PROFESSIONAL:

1.75

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

Tay-Sachs disease is an autosomal recessive disorder caused by mutations in the alpha-chain polypeptide of the A form of beta-hexosaminidase, a lysosomal enzyme composed of two chains. Such lesions result in a spectrum of disease ranging from severe to mild. The Ashkenazi Jewish population is enriched for carriers of a fatal form of Tay-Sachs disease. It had been presumed that Tay-Sachs patients from this ethnic isolate harbored the same alpha-chain mutation. We recently disproved this notion by identification of a splice junction defect in the alpha-chain of an Ashkenazi patient which could only be detected in 30% of the Ashkenazi carriers tested. We then isolated alpha-chain genomic clones from an Ashkenazi Jewish patient negative for the splice junction defect to determine this second mutation.

During the past year we completed sequence analysis of the promoter region, exon and splice junction regions and polyadenylation signal area of the mutant gene. We found a 4 base pair insertion in exon 11. This mutation introduces a premature termination signal in exon 11. We developed a dot blot assay utilizing the polymerase chain reaction to amplify the region of exon 11 encompassing the insertion to screen patients and carriers for the insertion mutation. The lesion was found in 70% of the carriers tested thereby distinguishing it as the major defect underlying Tay-Sachs disease in the Ashkenazi Jewish population.

In addition, we found that the DNA of the Ashkenazi Jewish patient used to isolate the alpha-chain clone bearing the insertion mutation was not homozygous for this mutation. These results indicated that a third mutation is probably present at very low frequency in the Ashkenazi population. We have isolated a lambda clone of this allele and are presently analyzing it to characterize the third lesion.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 18007-10 LEM

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Electrochemical Ion Gradients as a Mechanism of Cellular Message Transmission

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: E.F. Grollman Medical Officer (Research) LEM, NIDDK

Others: S. Doi Visiting Fellow LEM, NIDDK

COOPERATING UNITS (if any)

LEM, NIDDK (L.D. Kohn, G. Ashwell); NCI, DCBD (S. Shifrin); USUHS (D. Tombaccini); Smithsonian Institute (R.J. Montali); Univ. Sao Paulo (Medeiros-Neto)

LAB/BRANCH

Laboratory of Biochemistry and Metabolism

SECTION

Section on Cell Regulation

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.25

PROFESSIONAL:

2

OTHER:

0.25

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

The project involves the study of iodine metabolism in the thyroid. Prior studies used thyroid cells in culture to characterize properties of iodide transport, and described an iodoglycolipid that was an intermediate in iodine metabolism. (a) Studies over the last year focused on defects in iodine metabolism that lead to a decrease in thyroid hormone formation and enlargement of the thyroid (goiter). Three types of goiter were studied: i) goiter due to an iodine deficient diet, ii) goiter with a congenital defect in thyroglobulin synthesis with dyshormonogenesis, and (iii) defect in sialiation of thyroglobulin that could not be secreted into the thyroid follicle. Biochemical studies of thyroglobulin prepared from these goitrous thyroids revealed alternative pathways of thyroid hormone formation in the thyroid. Studies also involved chemical modifications of thyroglobulin. These studies use reagents that react selectively with lysyl residues. Thyroglobulin for these studies were the same as described above as well as from normal human and bovine thyroids. Treatment of thyroglobulin with these reagents releases low molecular weight peptides. Release of iodopeptides also occurs spontaneously. Since most thyroid hormones are contained in this area of the thyroglobulin molecule, the interaction of this peptide with thyroglobulin may have an important role in the synthesis and release of thyroid hormones from thyroglobulin. (b) Studies characterizing the iodide transport protein in the thyroid have continued, and take advantage of the similarity with the anion transport protein known as band 3 from erythrocytes. In prior studies, we showed that agents that specifically interact with band 3 also affect the transport of iodide by the thyroid. Monoclonal antibodies raised against band 3, also block iodide transport. We have now isolated several clones using a RNA expression library from thyroid cells, that hybridized with a cDNA probe to murine band 3. Studies have been initiated using these clones to express iodide transport activity in *Xenopus* oocytes.



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 18009-10 LEM

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Endocytosis, Secretion and Compartmentalization in Mutant CHO Cells

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	A.R. Robbins	Research Geneticist	LEM, NIDDK
Others:	C.W. Hall	Research Chemist	LEM, NIDDK
	S.M. Laurie	Visiting Associate	LEM, NIDDK
	C.F. Roff	Senior Staff Fellow	LEM, NIDDK
	T.M. Weber	Staff Fellow	LEM, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Biochemistry and Metabolism

## SECTION

Section on Enzymes and Cellular Biochemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

4

## PROFESSIONAL:

4

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- |   |  |   |
|---|--|---|
| <input type="checkbox"/> (a) Human subjects | <input type="checkbox"/> (b) Human tissues | <input checked="" type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors        |  |   |
| <input type="checkbox"/> (a2) Interviews    |  |   |

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Our approach to dissecting the processes of endocytosis, glycoprotein biosynthesis and sorting is through isolation and analysis of mutants. We had previously described two classes of endocytosis mutants in CHO cells, End 1 and End 2, which appeared to be defective in endosomal but not lysosomal acidification, based on assays of ATP-dependent acidification of organelles recovered from Percoll gradients. However, recent examination of living cells using the ACAS 470 indicated that lysosomal acidification also was decreased in these mutants. Pursuing this discrepancy we found that centrifugation through Percoll significantly reduces ATP-dependent acidification of lysosomes from normal cells. Using a modified labeling procedure whereby a pH reporter is compartmentalized only in lysosomes, we can assess lysosomal acidification without fractionation: we find that ATP-dependent lysosomal acidification in End 1 and 2 mutants decreases in parallel with endocytic activity.

We have obtained evidence for regulation of both the biosynthesis and compartmentalization of the Man6P/IGFII receptor. On return of a temperature-sensitive End 1 mutant from the non-permissive to the permissive temperature, we see rapid degradation of pre-existing receptor and rapid disappearance of receptor from the cell surface. However, the total number of Man6P/IGFII receptors remains constant due to a compensating increases in biosynthesis. By a yet to be defined mechanism these new receptors mix with the endocytic pathway at one-tenth the normal rate.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 18010-02 LEM

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Role of Intracellular Traffic in HIV Infection

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J.A. Hanover Senior Staff Fellow LEM, NIDDK  
Others: C. Starr IRTA LEM, NIDDK  
B. Wolff Visiting Associate LEM, NIDDK

COOPERATING UNITS (if any)

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INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.5

PROFESSIONAL:

1.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

CD4, the presumed T-cell receptor for the human immunodeficiency virus, depends upon glycosylation for proper surface expression. Tunicamycin, a potent inhibitor of glycosylation blocks expression of CD4 at the cell surface under conditions where alternate surface receptors are unaffected. Initial studies employing acute lymphoblastic leukemic cells have been extended by the successful transfection of a plasmid containing the cDNA for CD4 into mutant and wild type Chinese hamster ovary cells. Subsequent cotransfection with another plasmid containing the multiple drug resistant gene, has permitted the isolation of stable clones expressing large amounts of CD4. Characterization of these clones suggests that CD4 contains biantennary unsialylated complex-type oligosaccharides. The data also suggest that the inability of CD4 to reach the cell surface when it lacks carbohydrate is due to the selective degradation of unglycosylated CD4.

A targeting sequence has been identified which is sufficient to allow protein molecules as large as  $0.5 \times 10^6$  to pass through the nuclear envelope. This has been assessed in our laboratory by chemically coupling peptides having the nuclear localization sequence to a highly fluorescent cytoplasmic protein. The sequence Pro-Lys-Lys-Lys-Arg-Lys-Val-Cys is sufficient to target these conjugates to the nucleus. The tat and art gene products of HIV have sequences very similar to this targeting domain. In studies, we have begun to examine the structure of the nuclear pore complex, across which the many nucleocytoplasmic exchange processes must occur. The structure of the pore complex is explored using recombinant DNA techniques. The major nuclear pore glycoprotein np62 has been cloned, sequenced and expressed in cultured cells. The gene encoding this protein has been isolated and found to be 2.95 kb in length and devoid of introns. The availability of the primary sequence of this protein has allowed the preparation of anti-peptide antibodies which react with p62. These antibodies should prove useful for probing the function of the pore complex in the HIV life cycle.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 18011-02 LEM

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cell Specific Activity of Elements within the HIV-LTR

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: L. Hennighausen Visiting Associate LEM, NIDDK

Others: P.A. Furth Special Volunteer LEM, NIDDK

## COOPERATING UNITS (if any)

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## INSTITUTE AND LOCATION

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## TOTAL MAN-YEARS:

1.8

## PROFESSIONAL:

1.8

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

One clinical suggestion that HIV may be activated upon exposure to steroid hormones is the observation that HIV p24 antigenemia is more frequently found in pregnant rather than non-pregnant women and that this antigenemia disappears after parturition. Transcription from the HIV-LTR (1) was therefore studied in the presence or absence of hormonal stimuli in tissue culture cells and transgenic animals. In the presence of Dexamethasone transcription from the HIV-LTR in tissue culture cells was elevated at least 4-fold. Moreover expression from the HIV-LTR was elevated in transgenic elements during pregnancy suggesting that steroid hormones may contribute to HIV expression during pregnancy.

We are currently identifying the DNA sequences which mediate the steroid hormone action on the HIV-LTR and analyze their interaction with known transcription elements in the HIV-LTR.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 18012-05 LEM

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Direct Measurement of Forces between Membranes or Macromolecules

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

Pis:	V.A. Parsegian	Guest Researcher	LEM, NIDDK
	D.C. Rau	Expert	LEM, NIDDK

Others:	K. Gawrisch	Visiting Fellow	DCRT
	R. Podgornik	Visiting Fellow	LEM, NIDDK

## COOPERATING UNITS (if any)

Brock Univ., Ontario (R.P. Rand); Univ. British Columbia, Vancouver (E.A. Evans);  
 Univ. Minnesota (D.F. Evans); Princeton University (S.M. Gruner)

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## INSTITUTE AND LOCATION

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## TOTAL MAN-YEARS:

2.7

## PROFESSIONAL:

2.7

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

By directly measuring the forces between large molecules or between membranes we are learning finally how these interactions allow the formation of molecular assemblies. Beginning with the discovery that hydration or solvation forces are the most important interactions encountered during the last few nanometers approaching contact, we are now systematically investigating the molecular features that govern surface hydration. Hydration forces are very sensitive to relatively small changes in composition of lipid polar groups or of small molecules bound to the macromolecular surface.

Following our measurement of the change in molecular disorder accompanying changes in molecular interaction with separation, we have derived a model of molecular motion in the force field of neighboring molecules. There has finally been a connection established between molecular force and motion.

On the theoretical side also, we have analyzed the fluctuations of ionic charge and found that the resulting charge fluctuation forces, analogous to van der Waals forces, create strong attractive forces that explain the puzzling "long range hydrophobic interaction" between non-polar surfaces. Earlier ideas about this interaction had to assume improbable perturbation of water solvent hundreds of Angstroms from the surface.

In the study of lipid forces, we have succeeded in measuring the energies of non-lamellar lipid phases. It appears now that many cell membrane lipids that exist in lamellar form are actually under a very great level of bending strain having been forced from the curved conformation that they would otherwise prefer. Recognition of this strain has made clear that the incorporation of membrane proteins involves significant energies of protein lipid interaction of bending.

Most recently, we have begun to use NMR to detect the influence of membrane lipids on boundary water. It is becoming possible to make a connection between these resonance measurements and the directly measured forces between lipid bilayers.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

701 DK 18013-02 LEM

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Physics of Ionic Channels and other Proteins with Aqueous Cavities

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: V.A. Parsegian Guest Researcher LEM, NIDDK

Others: J. Kasianowicz IRTA LEM, NIDDK

C. Moore Special Volunteer LEM, NIDDK

D. Ruston Special Volunteer LEM, NIDDK

J. Zimmerberg Guest Researcher LEM, NIDDK

## COOPERATING UNITS (if any)

Rockefeller University (Sanford M. Simon, Ph.D., Gunther Blobel, Ph.D.); Hopkins University (Andrew Harris, Ph.D.); Wright State University (Anne Walter, Ph.D.); Office of Naval Research (Igor Vodyanoy, Ph.D.)

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## SECTION

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## INSTITUTE AND LOCATION

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## TOTAL MAN-YEARS:

2.2

## PROFESSIONAL:

2.2

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

We have been studying membrane transport proteins with regard to the physical factors that govern voltage gating and lipid protein interactions.

Beginning with the application of osmotic stress to determine the amount of water that enters an opening channel, finding that this amount is large enough to imply significant changes in protein arrangement, recognizing that such changes will in turn lead to different modes of protein packing in lipids, we are now varying the host lipids into which purified membrane proteins are incorporated during functional reconstitution. Preliminary results suggest that lipids affect not only voltage gating properties but also the ability of proteins to move into lipid bilayers.

We have developed a practical technique for incorporating a single protein into lipid vesicles that can be fused into planar bilayers for transport measurements. Differences in vesicular sedimentation often suffice to allow separation of vesicles containing different numbers or kinds of proteins.

We have found also that vesicles created from rough endoplasmic reticulum contain proteins that conduct electrical current when introduced into planar bilayers by vesicular fusion. This finding gives important support that protein translocation is associated with transport through pores.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 18014-05 LEM

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structure and Physical Properties of DNA and DNA-Protein Complexes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: D.C. Rau Expert LEM, NIDDK

## COOPERATING UNITS (if any)

George Mason University, Fairfax, VA (Dr. H. Chen); LMB, NIDDK (Dr. J. Nickol); LCP, NIDDK (Drs. M. Riehm and E. Charney); University of Nevada, Reno, Nevada (Dr. R. Harrington); Univ. of Calgary, Alberta, Canada (Dr. D. Bazett-Jones)

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## SECTION

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.7

## PROFESSIONAL:

.7

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Extending our previous results for myosin II monomer and dimer structure, we are now examining bipolar myosin filaments with electric birefringence. The orientation of the complex is entirely due to a field induced perturbation of the internal structure. We are able, therefore, to probe directly the dynamics of hinge (HMM-LMM junction) region motion. The precise role of hinge flexibility on force generation during the actin activated ATPase cycle of myosin is as yet unclear. We are now able to quantitatively relate hinge motion with ATPase activity. The limiting structure observed is consistent with a bipolar filament composed of 16 monomers. Hinge motion is best described by a bending spring rather than a free joint. Phosphorylation of the C-terminal control region dramatically changes the flexibility of the hinge region, presumably through the close proximity of hinge and control region on neighboring myosins. That phosphorylation also modulates ATPase activity strongly suggests that hinge motion is involved in force generation.

Within the past several years, it has become clear that DNA can adopt a variety of alternate B-form structures depending on sequence and ionic conditions. These structures may play a role in the organization of DNA in protein complexes, such as nucleosome positioning. The 5s RNA gene from sea urchin and *Xenopus* does form a uniquely positioned nucleosome. We have found that this DNA fragment also shows unusual flexibility with counterions that bind into grooves rather than to the sugar-phosphate backbone. This effect is not general for all DNA sequences and is due to either static bending or anisotropic flexing. Oligopurine stretches of length 5 bp or greater appear to be the affected sequences. The distribution of oligopurine sequences in these DNA fragments and anisotropic bending correlate well with nucleosome positioning.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 18015-05 IEM

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Histamine Release from Beige Mouse Mast Cells

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. Zimmerberg Guest Researcher LEM, NIDDK

Others: M.J. Curran Staff Fellow LEM, NIDDK

## COOPERATING UNITS (if any)

Rush Medical College, Chicago, IL (Fredric S. Cohen, Ph.D.), Arizona State University, Tempe, AZ (Douglas E. Chandler, Ph.D.)

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NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Mast cells of the beige mouse contain large intracellular secretory granules (approximately 4 microns in diameter) whose membranes fuse with the plasma membrane in a process called exocytosis. During membrane fusion an exocytotic pore forms which connects the granule interior with the extracellular medium. Through the exocytotic pore the granule contents are released extracellularly and are free to diffuse to target cells.

We have correlated electrophysiological and light microscopic data to investigate the structure of this exocytotic pore during secretion in mast cells from Beige mice. The time course for the widening of the pore is highly variable: it can widen quickly or slowly and can fluctuate between dilated and contracted states of variable conductance (flickering). Initial pore sizes are broadly distributed indicating that this pore is different from traditional membrane channels which have a relatively fixed conductance. The frequency histogram for occurrence of pores of given conductance is broad with a primary peak between 1 and 4 nS, indicating that pore size does not increase in quantal steps. A secondary maximum occurs at about 30 nS. We have searched fast-frozen, freeze-fracture replicas of rat mast cells and identified pores with small lumens. The 30 nS pores may represent the smallest pores seen in transmission electron microscopy. A model describing fusion on the molecular level which can account for the variable pore sizes, flickering, and known volumes of activation is described.

In a second project isolated matrices of the giant secretory vesicles of Beige mouse mast cells were examined to determine the effects of the ionic composition of the bathing solution on their size. In general multivalent cations condense the matrix relative to univalents.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 18016-02 LEM

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cell-Cell Fusion due to Influenza Hemagglutinin

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. Zimmerberg	Guest Researcher	LEM, NIDDK
	M.J. Curran	Staff Fellow	LEM, NIDDK

## COOPERATING UNITS (if any)

Laboratory of Theoretical Biology, NCI (R. Blumenthal, Ph.D., D. Kaplan, Ph.D., D. Sarkar, Ph.D.)

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## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- |   |  |   |
|---|--|---|
| <input type="checkbox"/> (a) Human subjects | <input type="checkbox"/> (b) Human tissues | <input checked="" type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors        |  |   |
| <input type="checkbox"/> (a2) Interviews    |  |   |

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We are continuing to study the infection process by which enveloped viruses, such as influenza, rabies, herpes and HIV, inject nucleic acids into the cytoplasm. This invasion is initiated via membrane fusion: the bound virus fuses to the plasma membrane or internalized membrane after receptor-mediated endocytosis. We study the fusion process directly by using a NIH3T3 fibroblast which expresses the influenza HA fusion protein in its surface. Thus the fibroblast surface has molecular features of the influenza virus, and will bind human red cells. Upon a change in external pH, the two cells fuse. We have measured fusion in three physically different ways: lipid exchange, cytoplasm exchange, and cell surface area change. These techniques indicate that influenza hemagglutinin-induced fusion rapidly establish both bilayer continuity and exchange of cytoplasmic contents. By using low light level video microscopy and image enhancement techniques, we followed the spatial relocation of both dyes in single cells during the fusion process. This study revealed the following new observations and insights into HA-induced cell fusion: (i) Continuous monitoring of fluorescence changes using two different criteria for fusion showed that fusion is rapid, the maximal extent is reached within minutes; (ii) The correspondence of the kinetics of the aqueous and lipid probes indicates that the cytoplasmic connections form as rapidly as the outer bilayers mix and there is no long-lived "partial-fusion" intermediate; (iii) Movement of fluorophores between effector and target was restricted during the initial events in fusion, consistent with the opening of small junctional pore(s); (iv) HA-induced leakage of a small molecule from the target occurred concomitant with fusion.

## ANNUAL REPORT OF THE LABORATORY OF CHEMISTRY

### NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

#### SECTION ON BIOCHEMICAL MECHANISMS

#### CHEMISTRY OF IMIDAZOLES AND BIOIMIDAZOLES

In various sections of this report, we describe significant and valuable applications of histidine analogues in biochemical and pharmacological studies. Such studies could have been performed many years ago, but for the fact that these analogues had not been available through classical or obvious synthetic routes. Even methods suitable for simple imidazoles may not be applicable to complex bioimidazoles, because of the additional functional groups and chirality. Thus, nonclassical methods (e.g., photochemical radical substitution, one-electron reduction, etc.) were developed to fit these gaps.

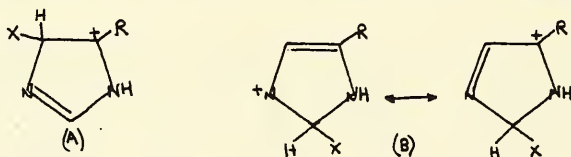
Even more novel methods are always being sought to provide analogues still inaccessible. We have now developed procedures for the conversion of amino-histidines into azido and nitrohistidine, of amino to chloro, bromo and iodo, of trifluoromethyl into methyl, cyano, carboxy, carbomethoxy, etc. Recently, we synthesized 2- and 4-(pentafluoroethyl)-histidines by photochemical radical substitution. These compounds are converted by base into the corresponding (trifluoroacetyl)-histidines, which have such reactive carbonyl groups that they may serve as affinity labels for histidine-binding sites. The trifluoroacetyl-imidazoles can be reduced to the secondary alcohols, also obtainable by direct condensation of imidazoles with trifluoroacetaldehyde. In turn, the secondary alcohols can be oxidized to the trifluoroacetyl ketones. Upon treatment with methanolic base, (trifluoromethyl)-histidine can be converted into (trimethoxymethyl)-histidine and pentafluoroethyl into the corresponding ketal. These ortho functionalities are also of interest as potential covalent affinity labels.

Ring-trifluoromethylated imidazoles show the unique property of losing hydrogen fluoride above pH 8 to form metastable difluorodiazafulvenes, which then react with any available nucleophile to form new covalent bonds. Such intermediates, derived from trifluoromethylhistamine or histidine, may be able to serve as covalent affinity labels for specific binding sites, both in vitro and in vivo. It would be desirable, therefore, to have available a series of trifluoromethyl analogues with a range of reactivities, and to be able to correlate reactivity with some substituent parameter. Our discovery of a simple photochemical method for the trifluoromethylation of imidazoles had made available a large series of analogues for study. We have found that the reactivities of some members of the group can be correlated with the special electronic effects of certain substituents (capable of hyperconjugation or back-bonding). Computer analysis of reactivity data for a series of trifluoromethyl-imidazoles has provided a linear free energy relationship in which log  $k_r$  correlates with both inductive and resonance components of the respective substituents. According to computer-based predictions, the fluoro group would provide the ideal combination of acidity and reactivity under physiological conditions. We have, therefore, developed procedures for sequential photochemical introduction of fluorine and trifluoromethyl into imidazoles and have verified the predicted reactivities. We are now involved in the preparation of peptide hormones containing these substituents. Photochemical introduction

of the trifluoromethyl group has been found practical for more complex imidazoles and studies are underway for the synthesis of the trifluoromethyl analogue of the anti-ulcer drug, cimetidine.

A number of 4-X-bioimidazoles are accessible by direct electrophilic substitution (nitro, halo); 2-X-bioimidazoles are far less accessible and can be obtained only by indirect and, often, very tortuous routes. Prior to our efforts in this area, the majority of 2-X-bioimidazoles were totally unknown. By far, the simplest 2-substituted bioimidazole now obtainable is the 2-iodo compound. 2-Iodo-L-histidine and 2-iodohistamine, unknown prior to 1985, are now accessible in large quantity by reduction of the corresponding 2,4-diiodo compounds in refluxing hydrochloric acid. Recent work has revealed, to our great surprise, that the same reductions can occur in hot water alone! Since 2,4-diiodo-5-methylimidazole does not show such behavior, we infer that the side-chain amino group plays the remarkable role of intramolecular, regiospecific reducing agent. 2-Iodohistidine is of interest not only because of its potent antimalarial activity (see later), but because it may serve as a valuable intermediate for the synthesis of other 2-X-histidines. We are currently exploring several schemes for such conversions.

From detailed mechanistic studies, we have determined that the specific removal for the 4-iodo group occurs by electrophilic replacement, involving attack by the hydronium ion and not the chloride ion (A). Thus, the same dehalogenation can be observed in the presence of any strong acid and any positive halogen acceptor (e.g.,  $\text{FeSO}_4$ ), and even in the absence of a halogen acceptor. The analogous dehalogenation at neutral pH may then involve regio-specific intramolecular proton transfer from the side-chain ammonium ion of protonated histamine or histidine. The remarkable preference for  $\text{S}_\text{E}$  exchange at C-4 over C-2 was largely predictable from our earlier studies of acid-catalyzed isotope exchange in imidazoles [JOC 44, 4210 (1979)]; the resistance to both isotope and halogen exchange at C-2 is attributable to the reluctance of the amidine carbon to surrender its resonance stabilization in order to achieve the required tetrahedral intermediate (B). The  $\text{S}_\text{E}$  mechanism requires



that the substituted imidazole be sufficiently nonbasic to provide a modest concentration of neutral species in a strongly acidic medium. This requirement is readily met not only by 2,4-dihaloimidazoles, but also by the more basic 4-haloimidazoles since 4-iodohistamine itself is readily dehalogenated. Thus, one can exclude the arguments that dehalogenation at C-4 requires the assistance of an electronegative substituent at C-2 in reducing nitrogen basicity or in stabilizing A. Nonspecific dehalogenation is observed in glacial acetic acid, apparently via a different mechanism. Such total specificity is also observed in the conversion of 2,4-dibromohistamine to 2-bromohistamine with refluxing HBr. With our discovery that 2-X-bioimidazoles prefer to exist as the natural 1,4-tautomer while 4-X-bioimidazoles exist as the unnatural 1,5-tautomer, the availability of synthetic routes to 2-X-imidazoles becomes particularly important (see next section).

A number of our 2-X-imidazoles are derived from the corresponding 2-amino-imidazoles. For the past 15 years, we have produced complex 2-aminoimidazoles by slow catalytic hydrogenolysis of 2-arylazoimidazoles. Unfortunately, guanidine resonance in 2-aminoimidazoles is sufficiently strong to effect significant dearomatization of the ring, and the 4,5-double bond now behaves like that of an isolated vinylamine. Thus, the double bond is readily reduced during hydrogenolysis of the azo function, or even with aqueous sodium borohydride. As a result, our hydrogenolysis step has never provided yields of 2-aminoimidazoles greater than 50%. Several years ago, we introduced the use of formamidinesulfonic acid [JOC 49, 1951 (1984)] to achieve the same reduction more efficiently for certain classes of 2-arylazoimidazoles. We have now found that transfer hydrogenation (ammonium formate, Pd-on-charcoal) is a far better technique, cutting reduction time from 7 days to 5 hours and eliminating production of any detectable dihydro products.

### ANTIMALARIALS

Our development, in 1971, of a photochemical route to ring-fluorinated aromatics and heteroaromatics has led to the synthesis of a wide variety of fluoro analogues of imidazole-based metabolites. Many of these compounds have shown interesting properties as agonists or antagonists and have proved useful as research tools and as possible chemotherapeutic agents. A striking difference has been found between 2-fluoro-L-histidine (2-FHIS) and the 4-fluoro isomer. While the former compound is readily incorporated into new protein in place of histidine (both in bacteria and mammals), the 4-fluoro isomer is not incorporated at all. Furthermore, 2-FHIS shows antibacterial, antiviral, antileukemic and antimalarial properties; again, the 4-fluoro isomer shows none of these activities. From our  $^{13}\text{C}$  NMR studies of other substituted histidines, we now suspect this differentiation to be based on tautomer preference in the imidazole ring; thus, 2-X-histidines resemble histidine in preferring the 1,4-tautomer, while 4-X and 2,4-di-X-histidines prefer the unnatural 1,5-tautomer. We found such differentiation in a variety of isomer pairs, including X = fluoro, iodo, trifluoromethyl. Furthermore, 2-fluorohistamine was found to be a potent agonist at the histamine H-1 receptor, while 4-fluorohistamine is active at the H-2 receptor. Again, tautomer preference may be the basis for such differentiation and may provide a rationale for the design of other agonists and antagonists.

We have become particularly interested in the antimalarial properties of 2-FHIS, since the compound is uniquely and selectively active against Plasmodium falciparum, that parasite which is notoriously resistant to chemotherapy. The organism has the unusual property of inducing production, within an invaded erythrocyte, of a protein containing as much as 70% histidine. The protein is found in "knobs" which are seen on the erythrocyte surface; these knobs are responsible for a very strong adherence of the infected erythrocytes to capillary endothelium, thereby sequestering parasitized cells which would normally be destroyed during passage through the spleen.

In cultures of infected erythrocytes, low concentrations of 2-FHIS not only inhibit cytoadherence but prevent maturation of the parasite and the appearance of knobs entirely. The assumption that these antiparasitic properties are due to the incorporation of 2-FHIS into the histidine-rich protein is probably unwarranted, since the treated parasite shows a general decrease in protein synthesis and rather low incorporation of  $^3\text{H}$ -2-FHIS. As one of several hypotheses for the mechanism of action, we propose that 2-FHIS interferes with

histidine as a promoter of the transport of some other amino acids into the cell. This hypothesis is supported by our earlier findings that 2-FHIS inhibits protein synthesis in cell and organ cultures but not in cell-free systems. Studies are in progress on the effect of 2-FHIS on facilitated amino acid transport. Unfortunately, the high antimalarial activity shown by 2-FHIS in vitro could not be extended, because the compound proved too toxic in monkeys. Such toxicity is surprising, since mice tolerate as much as 500 mg/kg. We are now exploring derivatives of 2-FHIS in the hope of reducing toxicity. Even if a less toxic analogue were found, the cost of large-scale production of 2-fluoroimidazoles would render them totally impractical. Despite our intensive efforts, small-batch photochemistry remains the only available route to fluoroimidazoles.

A large number of other substituted histidines have been screened for in vitro antimalarial activity: 2-iodo showed good activity while 2-azido showed moderate activity. Once again, 4-X-histidines show no activity. Surprisingly, the 2-chloro and 2-bromo analogues were inactive. Laboratory-scale production of 2-X-imidazoles, especially the histidines, is very time-consuming, involves multiple low-yield steps, and is limited to small batch operation. Our recent efforts to find alternative, and more economical routes have been successful - at least for 2-IHIS. Readily available 2,4-diiodo-L-histidine can be converted into mixtures of 2-IHIS, 4-IHIS and HIS by photoreduction, catalytic hydrogenation or reduction with titanium trichloride. The last method is especially promising, providing yields of 2-IHIS up to 20% in this one-step process. More recently, we have found that 2,4-diiodo-L-histidine can be reduced selectively with hot 3N HCl to 2-iodo-L-histidine, without formation of any of the 4-iodo isomer. Despite its promise in in vitro tests, the 2-iodo analog proved inactive in monkeys (but also nontoxic). It is likely that mammals possess a metabolic pathway for deiodination of the iodo compound. Deiodination of 4-iodohistidine in rats had been observed previously. Preliminary in vitro studies show that 2-iodohistidine is much less effective than 2-FHIS in interfering with protein synthesis and, thus, appears to operate by a different mechanism. We postulate that the iodo compound may be blocking nutrient diffusion holes in the erythrocyte membrane. The absence of an involvement in active transport channels is supported by our observation that 2-iodohistamine and its  $\alpha$ -N derivatives also show some in vitro inhibitory activity. Nothing is known about mammalian enzymes (tissue, substrate specificity, mechanism, or their very existence) capable of deiodinating iodohistamine or histidines. We have prepared the isomeric N-methyl-2-iodo analogues in the hope that N-alkylation may reduce or block the presumed deiodination process in vivo.

The role of the iodine atom may be steric or lipophilic (and probably not electronic). In order to attempt any structure-activity correlation, data is needed for other substituted histidines with large, lipophilic groups at C-2 (iPr, tBu, Ph, Bz, etc.). We are now developing new synthetic methods to obtain such compounds, based on (1) cyclization of dibenzoylaminoethylenes with acyl halides, (2) synthesis and hydrogenolysis of ketones and (3) imidazole-ring substitution with photochemically generated radicals. Strong efforts to develop method (1) have not been promising; method (2) has only limited applicability; preliminary studies with method (3) are very encouraging. Further clues to the design of effective antimalarials may be achieved from knowledge of the mechanisms of action of these histidine analogues. To this end, a synthesis of  $^{14}\text{C}$ -2-fluorohistidine has been developed. We have also demonstrated that H-4 in 2-iodohistidine can be exchanged with isotopic hydrogen under alkaline conditions.

## HYPOXIC CELL SENSITIZERS

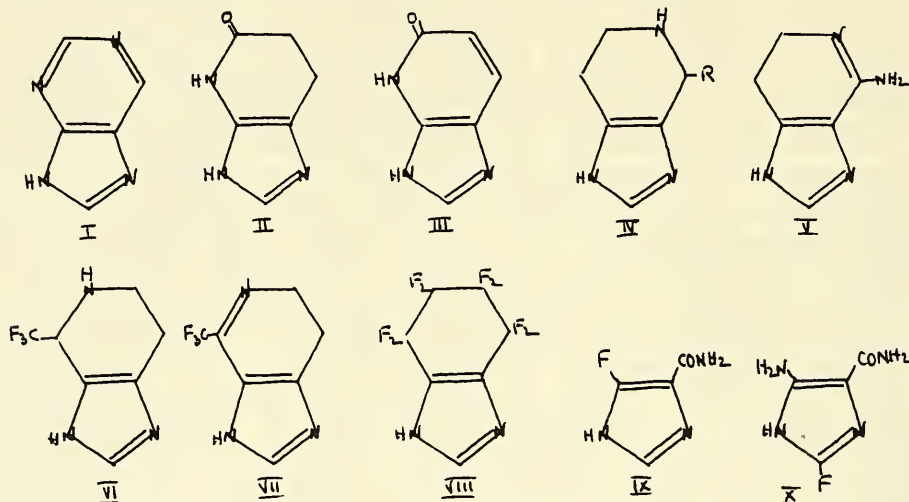
The valuable properties of nitroimidazoles as radiation sensitizers and as selective cytotoxic agents for cancer treatment have stimulated considerable research into mechanisms of action and metabolic fate of the drugs. We have proposed three theories for the mechanism of action: (1) Thiols are known to add to the 4,5-double bond of nitroimidazoles and, thus, such compounds may interfere with normal cellular functions by binding cysteine, glutathione, SH enzymes, etc. (2) Nitroimidazoles may be reduced in vivo, to hydroxylaminoimidazoles which can function as supernucleophiles in cleaving the phosphate ester bonds of polynucleotides; unfortunately, synthetic hydroxylaminoimidazoles have been found so unstable that their potential as nucleophiles cannot be investigated. As an alternative, we are devising synthetic methods for hydrazinoimidazoles; these compounds should be significantly more stable than hydroxylaminoimidazoles and, yet, should possess the same nucleophilic power inherent in hydroxylamine functions. Our primary interest is in 2-hydrazinoimidazoles: efforts to prepare these compounds by displacement of fluorine in 2-fluoroimidazoles have been unsuccessful; we are now trying reduction of 2-diazonium imidazoles with borohydride-metal combination. (3) Reduction of the nitro group by nonnucleophilic agents leads to nitro radicals; we believe these heterocyclic radicals capable of alkylating cell constituents and interfering with metabolism. To this end, we are now studying the anaerobic reduction of nitroimidazoles with one-electron transfer agents (e.g., titanous chloride). A critical factor in the development of new sensitizers is the availability of a practical synthesis of 2-nitroimidazoles. Although we have already published several routes, they are not truly practical and cannot be used with polyfunctional bioimidazoles. We are currently exploring methods involving nonselective radical nitration with photochemically generated nitro radical.

Misonidazole is an alkylated 2-nitroimidazole which has been found quite effective in sensitizing cancer cells to radiation and in reducing the radiation dose needed to effect significant cell destruction. Unfortunately, the compound has to be used at such high levels as to produce serious side effects and may not be released by FDA. We have postulated that the introduction of nitro groups into more natural imidazoles (histamine, histidine, etc.) may produce the desired alien molecule. Indeed, several such compounds have shown in vitro activity comparable to that of misonidazole. Evaluation of the clinical effectiveness of this series of compounds in animals is in progress.

## IMIDAZOLE ANTIVIRALS AND OTHER MEDICINALS

The notable success of virazole and deazapurine systems as antivirals has stimulated research into further modifications of the purine (I) ring system, especially those involving replacement of ring nitrogen with carbon. Analogues synthesized to date have required laborious multistep processes and have given only low yields. We have devised a number of simple syntheses which produce deazapurine analogues in good yield and with few steps. Reduction of 4-nitrohistidine ester or of 4-nitroimidazolepropionic ester leads to II. Reduction of 4-nitrourocanic ester gives the stable 4-aminourocanic ester, but subsequent irradiation converts the trans olefin to cis and the product cyclizes to III. Condensation of histamine with aldehydes gives series IV and cyclization of 4-(trifluoromethyl)-histamine with ammonia gives V. Series VI is obtained by condensation of histamine with trifluoroacetaldehyde, VII by cyclization of 4-(trifluoroethyl)-histamine with base, and VIII by photochemical reaction of imidazole with 1,4-diiodo-perfluorobutane. Compounds which we have previously

found to have significant antiviral activity (IX and X) are also being modified somewhat. Systems III-VII can be dehydrogenated to the fully aromatic systems with selenium dioxide. These compounds, with or without ribose attachment, will be evaluated for antiviral activity, particularly against AIDS.



A major program has been initiated to devise synthetic routes to hydrazinoimidazoles, which we consider promising antimetabolic agents. Such compounds are still unknown and predictions of stability and chemistry are based on the properties of phenylhydrazines. Thus far, efforts to replace fluorine in 2-fluoroimidazoles with hydrazine or to reduce 2-diazoniimidazoles have failed. A number of alternative approaches are still open and are being explored.

#### TRH ANALOGS

The simple tripeptide, L-pyroglutamyl-L-histidyl-L-proline amide (TRH), exerts marked cardiovascular, behavioral and analeptic effects, through activation of the sympathoadrenomedullary system. These effects appear to be unrelated to its action on the hypothalamo-pituitary axis to release thyrotropin and prolactin. Involvement of TRH in many nonendocrine functions of brain is also suggested by its distribution and the presence of high affinity binding sites outside the hypothalamus and pituitary. TRH has shown promise in the treatment of various forms of shock, as an analeptic, antidepressant and in promoting the regeneration of injured spinal cord. Practical clinical utility of the peptide is limited, however, by this very multiplicity of biological activities, as well as by its very low biological half life. The presence of degrading enzymes in blood serum, a difficulty in crossing the blood-brain barrier because of its polar structure, and the unavailability of facilitated or receptor-mediated transport - all serve to limit severely the survival of exogenous TRH and its delivery to the brain. On the other hand, the multiplicity of significant (or even vital) physiological activities of TRH argues strongly for the search for synthetic analogues which can not only overcome these limitations of stability and penetration but also achieve separation of the various activities.

The synthetic analogues used in our previous and current studies have all involved modification (or replacement) of the imidazole ring of histidine; these analogues have produced dramatic dissociation of some activities, suggesting that the different physiological functions of TRH may be mediated through different receptors or subtypes thereof. In contrast to TRH, 4-F-Im-TRH and 2-CF<sub>3</sub>-Im-TRH do not bind to pituitary GH<sub>4</sub> cells in vitro nor stimulate prolactin release from them; such results would immediately suggest the analogues to be nonfunctional. On the other hand, systemic injection or direct microinjection into rat brain of either analogue not only results in increased cardiovascular (CVS) effects (heart rate, blood pressure) comparable to those found with TRH, but also in release of prolactin at 2-3 times the level observed with TRH. In the whole animal, therefore, prolactin release can be controlled from receptor sites outside the pituitary. Enhanced CVS activity is also evident in 4-CF<sub>3</sub>-Im-TRH and 4-NO<sub>2</sub>-Im-TRH, and it would seem that the receptor for CVS activity is essentially indifferent to the position, size or nature of the imidazole ring substituent. The fallacy of this conclusion is demonstrated by the greatly reduced activity of 4-I-Im-TRH and the total inactivity of 2,4-I<sub>2</sub>-Im-TRH. Furthermore, replacement of histidine by an aliphatic amino acid (e.g., norvaline) also results in the loss of CVS activity. The spectrum of CVS and other activities are summarized in Table I.

Table I. Physiological Activities of TRH Analogues<sup>a</sup>

Compound <sup>b</sup>	CVS Activity	Prolactin Release	TSH Release <sup>c</sup>	CNS Activity <sup>c</sup>
TRH	+++	+	+++	+++
4-F-TRH	++	++ <sup>d</sup>		
4-CF <sub>3</sub> -TRH	+++	++		
2-CF <sub>3</sub> -TRH	+++	+++		
4-I-TRH	0(+)	+ <sup>e</sup>	0	
2,4-I <sub>2</sub> -TRH	0	+	0	
4-NO <sub>2</sub> -TRH	+++	0	0	
Nva <sup>2</sup> -TRH	0	+	0	+++ <sup>f</sup>

<sup>a</sup> By intra-arterial administration in conscious rats, unless otherwise indicated.

<sup>b</sup> Substitutions are all on imidazole ring of histidine.

<sup>c</sup> Reported in the literature.

<sup>d</sup> Both CVS and prolactin release are observed following central administration.

<sup>e</sup> Active at higher dose (30 µmol/kg).

<sup>f</sup> Ten times more potent than TRH in analeptic activity test.

It is evident from Table I that the structural requirements for CVS activity differ markedly from those for prolactin release and that, for the latter, an imidazole ring may not be necessary at all. Equally striking is the evidence that structure-activity factors for the release of thyrotropin-stimulating hormone (TSH) do not parallel those for prolactin release. The data suggest that in vivo elevation of prolactin by some of these analogues may not be the direct result of interaction with TRH receptors. An alternative mechanism may involve interaction of these analogues with the dopaminergic system. Dopamine is known to decrease levels of PRL and inhibition of dopamine release by these analogues could then explain the elevated levels of PRL. Experiments are being undertaken to test this hypothesis.

It is now quite clear that at least four of the biological activities of TRH involve uniquely different receptors and that, after decades of effort in various laboratories, the separation of these activities has at last been achieved. Thus, 4-NO<sub>2</sub>-TRH, highly selective for CVS activity, may be useful in the treatment of various forms of shock without a concomitant enhancement of thyroid activity or of prolactin release. On the other hand, 2,4-I<sub>2</sub>-Im-TRH or Nva<sup>2</sup>-TRH may be useful as diagnostic tools for the assessment of pituitary function without the risk of increased blood pressure and tachycardia induced by TRH. The iodinated analogue is particularly useful since it can be prepared readily with radioactive iodine. Furthermore, each of these selective agonists should provide a useful research tool for the study of the role and mechanism of TRH involvement in the respective function.

Receptor-binding studies have been completed for 15 analogues of TRH using rat pituitary, hypothalamus, brainstem and cortex tissues (Table II). The analogues show a wide range of affinities for the high affinity binding site of TRH receptors, but none bind in the nanomolar range characteristic of TRH itself. In fact, three analogues (4-CF<sub>3</sub>-TRH, 4(5)-NO<sub>2</sub>-TRH and 1-Me-4-NO<sub>2</sub>-TRH) show extremely low or no measurable binding. Nevertheless, 4-CF<sub>3</sub>-TRH and 4-NO<sub>2</sub>-TRH exhibit potent centrally mediated cardiovascular activity! It is therefore apparent that this activity is not mediated through the high affinity TRH receptor, but through a subtype which is not labelled by the radioligand 3-Me-His<sup>2</sup>-TRH. Careful receptor-binding studies of 4-NO<sub>2</sub>-Im-TRH with <sup>3</sup>H-TRH as radioligand have revealed that this analogue binds to a low affinity site, which is not labelled by [<sup>3</sup>H]-3-Me-His<sup>2</sup>-TRH but is labelled by <sup>3</sup>H-TRH. This site may be involved in mediating the cardiovascular effects of TRH and its analogues.

Three aspects of structure-binding relationship have emerged from these studies:

- 1) It appears that the binding of analogues is dependent on both electronegativity and size of the substituent. Computer-aided QSAR gave further insight into the interaction of the TRH analogues with TRH receptors. 4(5)-Br-TRH shows a significantly higher affinity than predicted by QSAR, suggesting that additional factors influence the binding of these analogues. We are investigating additional analogues to provide statistically significant data for a multiparameter regression analysis. The effect of substitution on tautomeric equilibrium and lipophilicity of the molecule are two important factors under investigation.

- 2) The fact that 2-CF<sub>3</sub>-TRH shows a 200-fold higher affinity than 4-CF<sub>3</sub>-TRH may be an important clue for inhibitor design, which has led us to the synthesis of other 2-substituted analogues. In particular, 2-Br-TRH is expected to show much higher binding and interesting biological consequences. Based on <sup>13</sup>C-NMR studies of 4- and 2-substituted histidines, it appears that the former exists as the  $\Pi$  tautomer and the latter as the biologically active  $\tau$  tautomer.

Table II. Mean values ( $\mu\text{M}$ ) for the inhibitor constants ( $K_i$ ) of TRH and its imidazole-substituted analogues  $\pm$  S.E. Experiments (number in parentheses) were done in triplicate each, using [ $^3\text{H}$ ]-3-Me-His $^2$ -TRH as radioligand.

Analogues (n)	Pituitary	Hypothalamus	Brain stem	Cortex
3-Me-TRH(2)	0.0034	0.0039	0.004	0.0066
TRH(4)	$0.019 \pm 0.001$	$0.033 \pm 0.004$	$0.026 \pm 0.010$	$0.048 \pm 0.013$
4-F-TRH(4)	$13.5 \pm 1.7$	$8.5 \pm 0.8$	$8.3 \pm 0.7$	$7.5 \pm 0.5$
4-Cl-TRH(2)	$39.7 \pm 8.7$	$32.6 \pm 5.5$	$23.1 \pm 3.4$	$26.2 \pm 2.9$
4-Br-TRH(4)	$2.5 \pm 0.09$	$1.93 \pm 0.15$	$1.59 \pm 0.06$	$1.81 \pm 0.19$
4-I-TRH(3)	$9.4 \pm 4.5$	$15.4 \pm 5.3$	$6.75 \pm 2.46$	$31.1 \pm 10.01$
2,4-I $_2$ -TRH(5)	$71.3 \pm 15.4$	$51.6 \pm 8.07$	$50.9 \pm 6.72$	$57.5 \pm 5.87$
4-CF $_3$ -TRH(2)	$569 \pm 40$	$465 \pm 148$	$1010 \pm 390$	$392 \pm 51$
2-CF $_3$ -TRH(4)	$4.1 \pm 1.40$	$3.25 \pm 0.18$	$3.65 \pm 0.27$	$4.94 \pm 0.12$
4-NO $_2$ -TRH(4)	$>1000.0$	$>1000.0$	$>1000.0$	$>1000.0$
4-CN-TRH(2)	$123 \pm 9.0$	$140 \pm 5$	$150 \pm 10$	$143 \pm 4$
4-CO $_2$ H-TRH (2-3)	$13.5 \pm 2.5$	$13.1 \pm 1.9$	$9.1 \pm 1.5$	$11.3 \pm 1.9$
2-CO $_2$ H-TRH (2-3)	$53.0 \pm 1.0$	$37.3 \pm 2.9$	$36.3 \pm 3.8$	$27.7 \pm 1.9$
Nva $^2$ -TRH(2-3) (2-3)	$19.9 \pm 3.9$	$29.2 \pm 7.9$	$20.1 \pm 5.4$	$72.8 \pm 2.4$
4-NO $_2$ -TRH(4)	$>1000.0$	$>1000.0$	$>1000.0$	$>1000.0$
1-Me-4-NO $_2$ -TRH (3)	$>>1000.0$	$>>1000.0$	$>>1000.0$	$>>1000.0$
1-Me-5-NO $_2$ -TRH (3)	5.0			

3) 4(5)-NO<sub>2</sub>-TRH, which is a selective cardiotoxic analogue of TRH, shows no binding to the high affinity TRH receptor ( $K_1 > 1 \text{ mM}$ ) and no TSH or PRL-releasing activity. This analog exists predominantly in the  $\Pi$  tautomer. In order to prove that tautomer preference plays a significant role in biological recognition, we prepared the frozen N-Me tautomer analogues: thus, 1-Me-4-NO<sub>2</sub>-TRH (simulating  $\Pi$ -histidine) does not bind to TRH receptors at all ( $K_1 \gg 1 \text{ mM}$ , and cannot be measured); on the other hand, 1-Me-5-NO<sub>2</sub>-TRH (simulating the biologically active  $\gamma$ -histidine) shows  $K_1 = 5 \text{ }\mu\text{M}$ , which is surpassed in affinity only by 4-Br-TRH ( $K_1 = 2.5 \text{ }\mu\text{M}$ ). The small effect of N-methylation in increasing the basicity of 4-NO<sub>2</sub>-TRH cannot explain this large increase in binding ability and, therefore, tautomerism must be a significant, if not predominant, factor in binding to the receptor. This finding has given us new leads for preparing high affinity ligands for TRH receptors. Thus, 1-Me-5-Br-TRH and 1-Me-2-Br-TRH should possess high affinity and are currently being prepared. Biological evaluation of these analogues should reveal interesting compounds and, possibly, the long sought antagonists of TRH.

The relatively high affinity for 1-Me-5-NO<sub>2</sub>-TRH ( $K_1 = 5 \text{ }\mu\text{M}$ ) and the expectation (based on QSAR studies) that transformation of the nitro group to azido (2 steps) will give even higher affinity has led to plans for the synthesis of the first photoaffinity label (1-Me-5-N<sub>3</sub>-TRH) for characterization of TRH-receptor systems.

A number of other new imidazole-modified analogues of TRH have already been prepared and others are in progress. With data on the pharmacology and neurobiology of all these analogues, we hope to identify the structural requirements and limitations for each type of activity, as well as the role of imidazole pK, aromaticity and hydrophobicity. In order to determine whether both ring nitrogens are necessary for activity and whether imidazole tautomers can be differentiated, we are currently preparing analogues of TRH with other heterocyclic rings in place of imidazole. Receptor-specific analogues will also be prepared with increased resistance to enzymic degradation and more lipophilic prodrugs are planned to accelerate penetration to the brain.

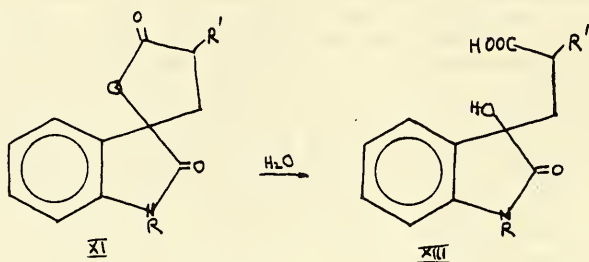
## CHEMISTRY, BIOCHEMISTRY AND PHARMACOLOGY OF BIOINDOLE ANALOGS

Tryptophan is an essential amino acid, serving as the precursor of the neurotransmitter, serotonin, and of the hormone, melatonin, in addition to its roles in enzymes and in receptor proteins. Tryptophan is metabolized in mammals by a pyrroloxygenase in the liver, where it can serve as a precursor of nicotinamide (Vitamin B) in some animals. In other tissues, tryptophan and related indoles are metabolized by a distinct oxygenase, the activity of which is dramatically increased (up to 100-fold) upon administration of bacterial lipopolysaccharides or interferon. The role of the oxygenase in the response of the organism to infection is unknown, however. We anticipated that certain 2-substituted tryptophans might serve as selective "suicide substrates" for these oxygenases. Analogs of tryptophan with electronegative substituents at C-2 had not been previously prepared. We have obtained 2-chloro and 2-bromo-L-tryptophan by radical halogenation, 2-trifluoromethyl-L-tryptophan by photochemical substitution, and 2-nitro-L-tryptophan as a minor product of direct nitration. Both the trifluoromethyl and nitro groups can be converted readily into other functions; some of these derivatives are of potential value as affinity and photoaffinity labels, as antibacterial agents and as photosensitizers in radiation therapy. 5-Azido-L-tryptophan has already been found effective as a photoaffinity label for tryptophan synthase.

The mechanisms of hydrolysis of the 2-halotryptophans at low pH have now been fully elucidated and reveal the involvement of intramolecular proton transfer in the conversion of the stable indole to the labile indolenine tautomer. An enzyme carboxyl group should also promote indolenine formation, suggesting the indolenine to be the true substrate for certain tryptophan enzymes. The first conclusive support for this concept is found in the demonstration that 2,3-dihydro-L-tryptophan and oxindolyl-L-alanine, analogs of the indolenine tautomer of tryptophan (tetrahedral carbon at C-3), are potent competitive inhibitors of tryptophan synthase and tryptophanase. Furthermore, the two enzymes show opposing specificity for the C-3 diastereoisomers of 2,3-dihydro-L-tryptophan suggesting that these enzymes catalyze their reactions via enantiomeric indolenine intermediates. The chiral center at C-3 in oxindolyl-L-alanine racemizes too readily to permit a study of opposing enzyme specificity. We have recently prepared the stable diastereoisomers of 3-hydroxy-oxindolyl-L-alanine (XIII) and, indeed, find the same opposing specificity for tryptophan enzymes as with dihydrotryptophan. The diastereoisomers of XIII have been identified by comparison with chiral synthons of the indole alkaloid, tryptoquivaline, whose absolute stereochemistry has been determined by x-ray crystallography. The inhibitory activity of XIII(R) on tryptophanase matches that of (3R)-2,3-dihydro-L-tryptophan. The lactone ring of XI is significantly strained and can be opened at C-3 by nucleophilic displacement. Thus, the 3-cyano and 3-azido analogs of XIII have now been prepared for study as inhibitors. In particular, the 3-azido analog may serve as a photoaffinity label. Reaction of XIII with DAST (diethylaminosulfur trifluoride) converts the 3-hydroxyl group into fluorine. Unfortunately, the fluorine atom appears to be far more reactive than anticipated, and recyclization to XI occurs almost without provocation.

In the course of deblocking the diastereoisomers of XI ( $R = H$ ,  $R' = \text{NHCbz}$ ) to the corresponding aminolactones, we were surprised to observe a facile racemization at C-3. This phenomenon occurs either with  $\text{HBr/AcOH}$  or with trifluoroacetic acid and reveals the facile generation of a carbocation at C-3. Theoretical considerations had predicted that the magnitude of destabilization of the carbocation by the adjacent carbonyl (C-2) should outweigh that of stabilization by the benzene ring. These expectations, however, are contradicted by experiment. Thus, it became necessary to develop alternative and less direct routes to the diastereoisomeric aminolactones. Such racemization has not been

observed with the hydroxyacids (XIII), suggesting the need for the superior leaving ability of the acylated 3-hydroxyl group or a dependence on the ring strain present in the lactone ring of XI.



### TENUTAUTOMER ANALOGS

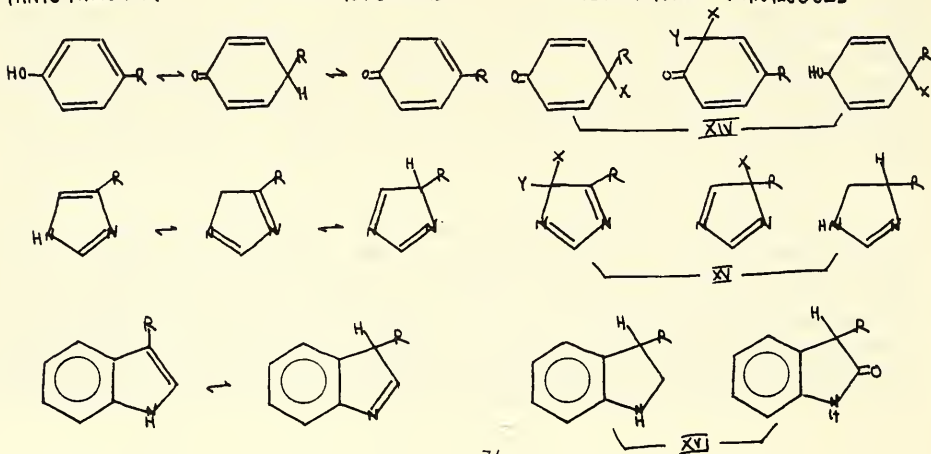
Our studies in tryptophan chemistry and biochemistry have revealed that the molecules present in the active sites of tryptophanase and tryptophan synthase are not the common NH tautomers (tantotautomers) of tryptophan but the higher energy, minor tautomers (tenutautomers). A variety of biological metabolites have similar major and minor capabilities - phenols, catechols, imidazoles, purines, etc. It is conceivable, therefore, that a variety of enzymes utilize an ability to bind and stabilize tenutautomers as a means of activating the substrate for a chemical transformation. We now have ample evidence that tenutautomers are the active species in a number of test-tube reactions of both phenols and imidazoles; furthermore, the experimental data for some enzyme-catalyzed reactions might become more intelligible if the substrates were viewed as their tenutautomers.

Since it is still impossible to examine the detailed structure of a substrate within a binding site, arguments for the tenutautomer concept must be based on evidence and inference from the behavior of stable tenutautomer analogues (XIV, XV). This approach was highly successful and very convincing in the case of tryptophan (XVI). We have undertaken analogous studies for the other tautomeric systems.

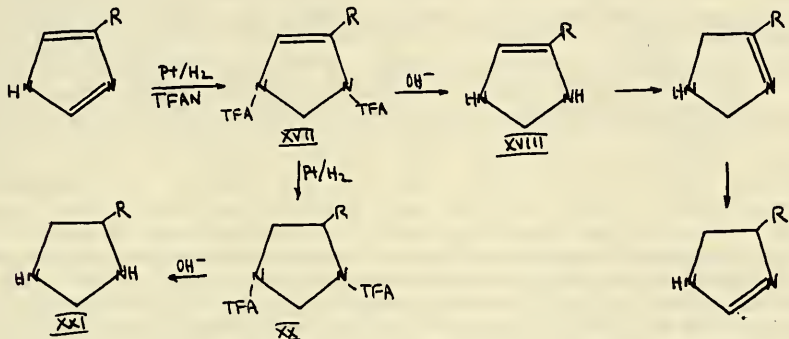
#### TANTOTAUTOMER

#### TENUTAUTOMERS

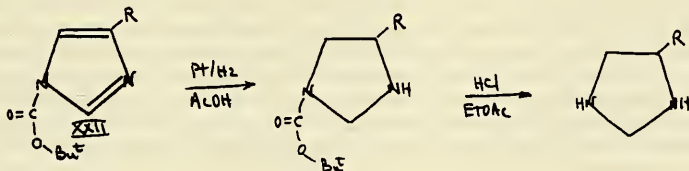
#### TENUTAUTOMER ANALOGUES



The imidazole ring is considerably more refractory to reduction than even benzene. We have found that catalytic hydrogenation can be achieved in the presence of trifluoroacetic anhydride, leading to the acylated dihydro derivative (XVII). The trifluoroacetyl groups of XVII can be removed at pH 12-13 to give the free amino acid (XVIII). We then hope to isomerize the double bond of XVIII to form XIX. Continued reduction of XVII gives the fully reduced ring. Both XVIII and the tetrahydro derivative of histidine are expected to serve as inhibitors of tetrahydrofolate reductase.



In order to avoid the problems associated with removal of the trifluoroacetyl groups, as well as to extend this method to histidine reduction in peptides, we have demonstrated that t-butoxycarbonylation and protonation are sufficient to activate the imidazole ring for reduction. Thus, XXII undergoes hydrogenation with platinum in glacial acetic acid to give the tetrahydro derivative. The mildness of this procedure now permits application to peptide hormones.



## GENERAL PRINCIPLES OF ENZYME CATALYSIS AND SIMULATION

In order to account for the remarkable catalytic power of enzymes, it is generally considered that the activation free energy (the energy hill which must be surmounted to get from starting material to product) is contributed both by binding of the substrate to the enzyme (step 1) and by chemical manipulation of bound substrate (bond-making and breaking, step 2). Popular opinion holds that most of the activation energy is supplied in step 2; We have proposed, however, that the overall catalytic process can be explained more reasonably if it is assumed that the first step (binding) contributes a more significant, and sometimes major, share of the activation energy. To support this theory, we have synthesized a large variety of test-tube models which simulate the bound substrate by being frozen into a single, favorable conformation and by having the interacting groups brought into the closest possible juxtaposition (stereopopulation control). The compounds undergo intramolecular reactions at rates comparable to those catalyzed by enzymes, sometimes even too fast to measure. Enzymes catalyze many reactions which cannot be observed under mild laboratory conditions. We have shown that our "locked" test-tube analogs can undergo a number of these reactions under physiological conditions of temperature and pH. Thus, one can demonstrate such difficult processes as hydride transfer and displacement of aromatic halogens. Recent work has involved the synthesis of compounds designed (1) to evaluate the flexibility of conformationally frozen carbon chains by ring-ring interconversion and (2) to study steric and electronic effects on  $^1\text{H}$  and  $^{13}\text{C}$  nmr spectra through space rather than through covalent bonds. Studies with several series of aromatic systems have shown that both reaction kinetics and spectral properties are linearly related to the van der Waals size of crowding substituents. The upper limits of energy-related steric crowding could not be evaluated, however, because of inability to introduce the very bulky iodo substituent. After considerable effort, this goal has now been reached; kinetic and spectral studies are in progress.

As part of our studies of practical applications of stereopopulation control, we are currently exploring the use of o-nitroaryl derivatives of biogenic amines and antibiotics as prodrugs. The intent is to facilitate passage from gut to circulatory system and from circulatory system to brain by temporary masking of charge within the molecule. A number of neutral derivatives of dopamine and L-dopa have been synthesized and are undergoing evaluation as prodrugs for penetration of the blood-brain barrier and as slow-release precursors in the blood stream. Protection from premature oxidation of the catechol function and increased lipophilicity have been introduced by use of the cyclic phenylboronate ester. Model studies show that the boronate is gradually cleaved under physiological conditions. In order to increase the oral effectiveness of polar antibiotics, similar nitroaryl protecting groups are being explored. Several such analogs of penicillin have been synthesized and will be submitted for evaluation.

## SECTION ON CARBOHYDRATES

The Section continues its work on the interaction of antigens and monoclonal antibodies. The elucidation of this interaction in great molecular detail is important because it throws light on the interaction of determinants with proteins in general. Thus it pertains to drug-receptor, effector-receptor, as well as viral-receptor interactions. The objective is approached simultaneously in different ways:

1. The physico-chemical study of the interaction between proteins and antigens.
2. The organic synthesis of complex ligands for affinity labelling and evaluation of specific areas of epitopes involved in binding.
3. The manipulation of immunoglobulin genes towards the production of site-specifically altered immunoglobulins for study.
4. The binding of carbohydrate epitopes to their monoclonal antibodies in order to investigate, by NMR techniques, the detailed conformational changes occurring in that process.
5. The preparation of immunodeterminants, followed by the preparation of specific antisera for evaluation.

### RECENT WORK

#### Sub 1.

Our work on the solution-interaction of monoclonal anti-dextran immunoglobulins (mAbs) with synthetic deoxy- and deoxyfluoro-glucosyl oligosaccharides is continuing. One of these mAbs had its sequence and three dimensional model reported by others. Using our solution-data on the binding of many of our synthetic ligands, Drs. G. R. Perdomo, and P. Kovac in the Section were able to make a unique fitting of the terminal glucosyl residue of the dextran antigen in the antibody's highest affinity subsite. This fitting had a degree of detail which equals that found by high resolution x-ray diffraction studies, and shows the power of our method (this work has been submitted to *Proc. Nat. Acad. Sci.*)

Other mAbs with dextran specificity are being isolated from ascites fluids, and purified. These are antibodies with differing binding patterns. We will elucidate the subsite arrangement these Abs have for the dextran epitopes they bind. Thus our laboratory will have been able to present a comprehensive picture of epitope-antibody binding. This work is in progress in collaboration with Dr. Elvin A. Kabat at Columbia University.

#### Sub 2.

A large number of mono- and oligo-saccharides in the (1→6)-dextran series, all bearing deoxy- and deoxyfluoro groups have been prepared by Dr. P. Kovac, and the results have been published in *Carbohydrate Research*. In addition, Dr. E. Nashed has extended her synthetic approach to include a new method for glycoside synthesis. This method allows the stereoselective synthesis of two saccharide units, one protected at C-1 and the other at C-6 with trimethylsilyl and *tert*-butyldiphenylsilyl groups, respectively. The reaction is carried out in the presence of trimethylsilyl triflate, and gives  $\beta$ -oligosaccharides in good yields. The work has been submitted for publication to *J. Org. Chem.* Dr. Th. Ziegler, a special volunteer from the University of Stuttgart, has been working on the preparation of large oligosaccharides in the (1→6)- $\beta$ -linked galactosyl series, in order to study binding modes of antigalactan mAbs. That work will be presented at the Gordon Conference this year.

#### Sub 3.

The cloning of genes in the galactan binding immunoglobulin repertoire is continuing. Due to the abundance of restriction sites in the heavy-chain gene, we have encountered difficulties in obtaining the suitable fragment, but it appears now that Dr. A. S Rao has overcome these difficulties. Thus, we have now in hand the light-chain gene and the heavy-chain gene of IgA X24, and are ready for their production in quantity. Following that the oligonucleotide-directed mutagenesis should be easy going.

Sub 4.

It was shown for the first time that the solution-conformation of a carbohydrate epitope may *not* be the conformation of that same epitope when bound to its monoclonal antibody. Using deuterio-, and fluoro-substituted disaccharides in order to identify the NMR signals, we have been able to show that the intersaccharidic angles of a (1→6)- $\beta$ -linked digalactoside change significantly when that ligand is bound to its mAb. This work, by special volunteer Dr. G. D. Daves and others, in collaboration with Dr. A. Bax, will be submitted to *SCIENCE*.

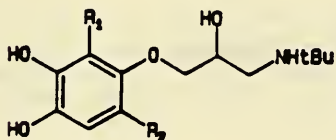
Sub 5.

Oligosaccharides occurring on glycoproteins, were previously synthesized in the Section by Dr. G. Ekborg. Some of these, which are the same as the saccharides occurring in the glycoprotein of the HIV, have been linked to Keyhole Limpet Hemocyanin and Bovine Serum Albumin, and we have prepared rabbit antisera against some of these. The antigens and antiserum are being evaluated for their capability to bind to T-cells and HIV respectively. This work is done in collaboration with Dr. S. Schnittman in NIAID.

## SECTION ON DRUG RECEPTOR INTERACTIONS

### Agonist Properties of Fluorinated Biogenic Amines.

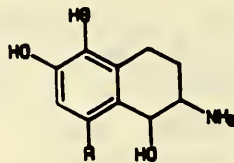
1-t-Butyl-1-(2-hydroxy-3-(3,4-dihydroxyphenoxy)propyl), 3a, is a potent  $\beta$ -adrenergic agonist. Our demonstration that 3b, the 6-fluoroanalog of 3a, has greatly reduced  $\beta$ -adrenergic agonist activity relative to 3a, while 3c, the 2-fluoroanalog has adrenergic behavior comparable to the parent 3a, suggested that a specific interaction of fluorine with the side-chain hydroxyl group may not be critical in defining adrenergic selectivities. We have examined the adrenergic activity of 6,7-dihydroxy amino tetralols (4a) and the corresponding fluorinated analog (4b). The parent tetraol 4a has been shown to be a  $\beta$ -adrenergic agonist, confirming other results that show that amines having this particular side-chain conformation interact effectively with the  $\beta$ -adrenergic receptor. We now report that the fluorinated analog 4b has greatly reduced  $\beta$ -adrenergic activity, a result that further suggests that the ability of fluorine to influence adrenergic activity is not related to side-chain conformation, since the conformation also is fixed in 4b. These results are consistent with the results obtained with 3a, 3b, and 3c.



3A.  $R_1 = R_2 = H$

3B.  $R_1 = H, R_2 = F$

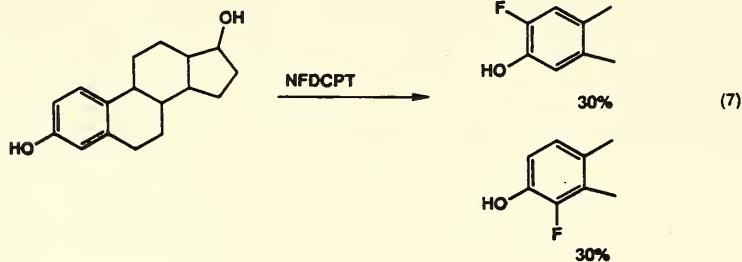
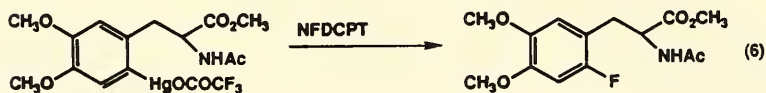
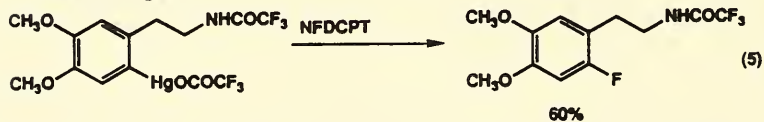
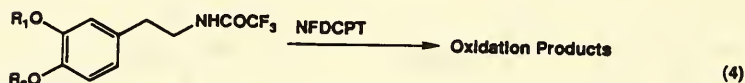
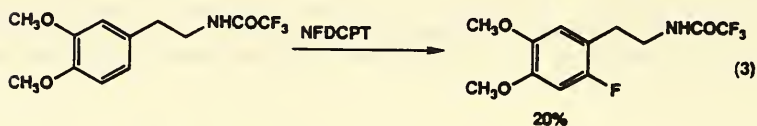
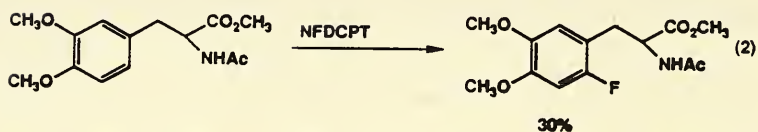
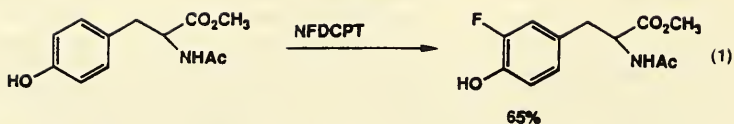
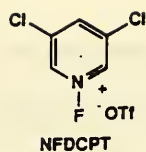
3C.  $R_1 = F, R_2 = H$



(4a)  $R = H$   
(4b)  $R = F$

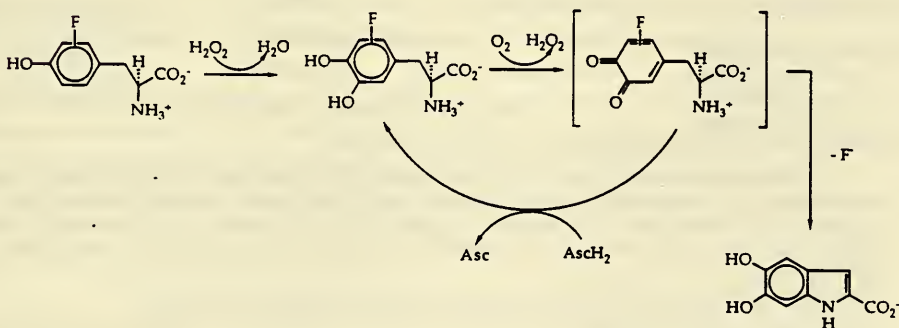
### Fluorinated Analogs as PET-scanning Agents. As part of our

continuing research on the development of  $^{18}F$ -labelled analogs as PET-scanning agents, we have examined new syntheses of fluorine-labelled amines, amino acids, and other naturally occurring compounds using new electrophilic fluorinating agents. The recently described N-fluoro-3,5-dichloropyridinium triflate (NFDCPT, 5) was prepared and the ability of this reagent to effect the fluorination of several phenolic substrates was examined. The results are summarized in reactions 1-7 below. It can be noted that while fluorination of a simple phenol is facile, catecholic systems give only oxidation products unless both of the oxygens are protected. Electrophilic demercuration with 5 gives improved yields relative to direct fluorination (Reactions 5-6).



Fluorinated Analogs as Potential Chemotherapeutic Agents. Our demonstration that 6-fluoroanalogs of DOPA, dopamine, and norepinephrine suffer release of fluoride during oxidation to bicyclic quinones (melanin precursors) prompted us to consider this as a means to deliver potentially toxic fluoride (or other anions) selectively to cells having high tyrosinase activity. As an extension to this approach we had synthesized 2-fluorotyrosine as a possible precursor to 6-fluoro-DOPA in vivo. Since two products are possible from enzymatic hydroxylation of 2-fluorotyrosine (2-fluoro- and 6-fluoro-DOPA), depending on the regioselectivity of this oxidation, we have studied 2-fluorotyrosine (and 3-fluorotyrosine) as substrates for mushroom tyrosinase. While 3-fluorotyrosine produced 5-fluoro-DOPA as predicted, 2-fluorotyrosine gave exclusively 6-fluoro-DOPA accompanied by rapid fluoride release, even in the presence of ascorbate. These results suggest that rapid cyclization of nascent 6-fluorodopaquinone occurs, competitively with reduction by ascorbate (Scheme 1).

Scheme I  
Proposed Reaction of Fluorotyrosines with Tyrosinase



## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK19001-17 LC

## PERIOD COVERED

October 1, 1988 to September 30 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

## Reactions and Immunochemistry of Carbohydrates

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal investigator.) (Name, title, laboratory, and institute affiliation)

PI: Cornelis P. J. Glaudemans, Chief Section on Carbohydrates, NIDDK-LC

Others:	P. Kovac	Research Chemist
	E. Nashed	Visiting Associate
	V. Pavliak	Visiting Fellow
	G. R. Perdomo	Visiting Associate
	A. S. Rao	Visiting Scientist
	Th. Ziegler	Special Volunteer

## COOPERATING UNITS (if any)

Elvin A. Kabat, Columbia University  
S. Schnittman, NIAID

LAB/BRANCH Laboratory of Chemistry

SECTION Section on Carbohydrates

INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

PROFESSIONAL: 5

OTHER:

## CHECK APPROPRIATE BOX(ES)

- |   |  |   |
|---|--|---|
| <input type="checkbox"/> (a) Human subjects | <input type="checkbox"/> (b) Human tissues | <input checked="" type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors        |  |   |
| <input type="checkbox"/> (a2) Interviews    |  |   |

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The Section continues its work on the interaction of antigens and monoclonal antibodies. The elucidation of this interaction in great molecular detail is important because it throws light on the interaction of determinants with proteins in general. Thus it pertains to drug-receptor, effector-receptor, as well as viral-receptor interactions. The objective is approached simultaneously in different ways:

1. The physico-chemical study of the interaction between proteins and antigens.
2. The organic synthesis of complex ligands for affinity labelling and evaluation of specific areas of epitopes involved in binding.
3. The manipulation of immunoglobulin genes towards the production of site-specifically altered immunoglobulins for study.
4. The binding of carbohydrate epitopes to their monoclonal antibodies in order to investigate, by NMR techniques, the detailed conformational changes occurring in that process.
5. The preparation of immunodeterminants, followed by the preparation of specific antisera for evaluation.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 19003-02 LC

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Synthesis of Immunodeterminants

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Pavol Kovac Research Chemist NIDDK-LC

Others: C. P. J. Glaudemans Chief, Section on Carbohydrates NIDDK-LC  
G. Perdomo Visiting Associate NIDDK-LC

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Chemistry

## SECTION

Section on Carbohydrates

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

This project has been incorporated into Project No. Z01 DK 19001-17 LC.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 19603-13 LC

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Histidine Analogs

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Louis A. Cohen Chief, Section on Biochem. Mech. NIDDK-LC

Others: N. Kolodny Visiting Fellow (Term 12/31/88) NIDDK-LC  
 S. Von Hof Visiting Fellow NIDDK-LC  
 B. Avramovitch Visiting Fellow NIDDK-LC  
 A. D'sa Visiting Fellow (Arr. 3/13/89) NIDDK-LC

COOPERATING UNITS (if any) G. Feuerstein, USUHS; E. DeClercq, Louvain, Belgium; W. Nagai, Nagoya, Japan; H. Kimoto, Nagoya, Japan; R. Howard, Palo Alto, CA; V. Labroo, USUHS; A. Faden, San Francisco, CA; W. Dürckheimer, Frankfurt, FRG; A. Oduola, AFIP; C. Bankiewicz, NINDS; S. Avramovici, Jerusalem, Israel

## LAB/BRANCH

Laboratory of Chemistry

## SECTION

Section on Biochemical Mechanisms

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.6

## PROFESSIONAL:

2.4

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

TRH Analogs: In addition to governing the release of thyrotropin and prolactin in the pituitary gland, TRH (L-pyrroglutamy-L-histidyl-L-proline amide) is known to possess a wide variety of effects on both the central nervous system (CNS) and the cardiovascular system (CVS). TRH has shown promise for use in the treatment of shock, as an analeptic and antidepressant, and as a promoter of the regeneration of injured spinal cord. However, the great variety of its biological effects presents a serious drawback to its use as a specific drug. Our early studies with synthetic analogs of TRH (involving modification of the imidazole ring of histidine) have suggested that the peptide hormone elicits each of its physiological responses at a different receptor and that appropriate analogs may achieve some of the desired specificity of action.

It is now quite clear that at least four of the biological activities of TRH involve uniquely different receptors and that, after a decade of effort in various laboratories, the separation of these activities has at last been achieved. Thus, 4-NO<sub>2</sub>-IM-TRH, high selective for CVS activity, may be useful in the treatment of various forms of shock without a concomitant enhancement of thyroid activity or of prolactin release. On the other hand 2,4-I<sub>2</sub>-Im-TRH or Nva<sup>2</sup>-TRH may be useful as diagnostic tools for the assessment of pituitary function without the risk of increased blood pressure and tachycardia induced by TRH. The iodinated analog is particularly useful since it can be prepared readily with radioactive iodine. Furthermore, each of these selective agonists should provide a useful research tool for the study of the role and mechanism of TRH involvement in the respective function.

Binding studies in rat brain tissue show that these analogs bind only weakly or not at all. We now have strong evidence that these analogs operate through non-TRH receptors to produce their CVS and CNS effects. Computer-assisted structure-activity analysis helps us design the most selective and potent analogs.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 19604-19 LC

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

General Principles of Enzyme Catalysis and Simulation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Louis A. Cohen

Chief, Sec. Biochem. Mech.

NIDDK-LC

Other: Michael King

Special Volunteer

GWU

## COOPERATING UNITS (If any)

Yoshio Ueno, Nagoya, Japan; Wieslaw Antkowiak, Poznan, Poland;

Yoshio Takeuchi, Toyama, Japan; Walter Dürckheimer, Frankfurt, FRG

## LAB/BRANCH

Laboratory of Chemistry

## SECTION

Section on Biochemical Mechanisms

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.2

## PROFESSIONAL:

0.2

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In order to account for the remarkable catalytic power of enzymes, it is generally considered that the activation free energy is contributed both by binding of the substrate to the enzyme (step 1) and by chemical manipulation of the bound substrate (bond-making and breaking, step 2). Popular opinion holds that most of the activation energy is supplied in step 2: We have proposed, however, that the overall catalytic process can be explained more reasonably if it is assumed that the first step (binding) contributes a more significant, and sometimes major, share of the activation energy. To support this theory, we have synthesized a large variety of test-tube models which simulate the bound substrate by being frozen into a single, favorable conformation and by having the interacting groups brought into the closest possible juxtaposition (stereopopulation control). These compounds undergo intramolecular reactions at rates comparable to those catalyzed by enzymes, and show that the protein raises both the entropic and enthalpic components of the substrate by binding it in a single, rigid conformation. Recent work has involved the synthesis of compounds designed (1) to evaluate the flexibility of conformationally frozen carbon chains by ring-ring interconversion and (2) to study steric and electronic effects of  $^1\text{H}$  and  $^{13}\text{C}$  nmr spectra through space rather than through covalent bonds. Studies with several series of aromatic systems have shown that both reaction kinetics and spectral properties are linearly related to the van der Waals size of crowding substituents. The upper limits of energy-related steric crowding could not be evaluated, however, because of inability to introduce the very bulky *isopropyl* substituent. After considerable effort, this goal has now been reached; kinetic and spectral studies are in progress. As part of our studies of practical application of stereopopulation control, we are currently exploring the use of *o*-nitroaryl derivatives of biogenic amines and antibiotics as prodrugs. The intent is to facilitate passage from gut to circulatory system to brain by temporary masking of charge within the molecule.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 19605-13 LC

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chemistry of Substituted Imidazoles

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Louis A. Cohen

Chief, Section on Biochem. Mech.

NIDDK-LC

Others: B. Avramovitch Visiting Fellow

NIDDK-LC

COOPERATING UNITS (if any) H. Kimoto, Nagoya, Japan; R. Henkin, Georgetown Univ. Hosp., Wash., D.C.; E. DeClercq, Louvain, Belgium; A. Shanzer, Rehovot, Israel; W. Nagai, Nagoya, Japan; S. Avramovici, Jerusalem, Israel; V. Labroo, USUHS; J. Retey, Karlsruhe, FRG; A. Phillips, Univ. Park, PA; W. Dürckheimer, Frankfurt, FRG.

## LAB/BRANCH

Laboratory of Chemistry

## SECTION

Section on Biochemical Mechanisms

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

0.8

## OTHER:

0.2

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A number of 4-X-bioimidazoles are accessible by direct electrophilic substitution (nitro, halo); 2-X-bioimidazoles are far less accessible and can be obtained only by indirect and, often, very tortuous routes. Prior to our efforts in this area, the majority of 2-X-bioimidazoles were totally unknown. By far, the simplest 2-substituted bioimidazole now obtainable is the 2-iodo compound. 2-Iodo-L-histidine and 2-iodohistamine, unknown prior to 1985, are now accessible in large quantity by reduction of the corresponding 2,4-diiodo compounds in refluxing hydrochloric acid. Recent work has revealed, to our great surprise, that the same reductions can occur in hot water alone! Since 2,4-diiodo-5-methylimidazole does not show such behavior, we infer that the side-chain amino group plays the remarkable role of intramolecular, regiospecific reducing agent. 2-Iodohistidine is of interest not only because of its potent antimalarial activity, but because it may serve as a valuable intermediate for the synthesis of other 2-X-histidines. We are currently exploring several schemes for such conversions.

A number of our 2-X-imidazoles are derived from the corresponding 2-aminoimidazoles. For the past 15 years, we have produced complex 2-aminoimidazoles by slow catalytic hydrogenolysis of 2-arylaazoimidazoles. Unfortunately, guanidine resonance in 2-aminoimidazoles is sufficiently strong to effect significant dearomatization of the ring, and the 4,5-double bond now behaves like that of an isolated vinylamine. Thus, the double bond is readily reduced during hydrogenolysis of the azo function, or even with aqueous sodium borohydride. As a result, our hydrogenolysis step has never provided yields of 2-aminoimidazoles greater than 50%. Several years ago, we introduced the use of formamidinesulfinic acid [JOC 49, 1951 (1984)] to achieve the same reduction more efficiently for certain classes of 2-arylaazoimidazoles. We have now found that transfer hydrogenation (ammonium formate, Pd-on-charcoal) is a far better technique, cutting reduction time from 7 days to 5 hours and eliminating production of any detectable dihydro products.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 19606-13 LC

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Halogenated Biogenic Amines in Biochemistry and Pharmacology

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Kenneth L. Kirk	Research Chemist	NIDDK-LC
Other: Adeboye Adejare	Visiting Fellow	NIDDK-LC
David Hebel	Visiting Fellow	NIDDK-LC
Kenneth A. Jacobson	Research Chemist	NIDDK-LC
Hauh-Jyun Candy Chen	Visiting Fellow	NIDDK-LC
Silvia Calderon	Guest Worker	NIDDK-LC
George Chen	Guest Worker	NIDDK-LC
Vital Shetty	Guest Worker	NIDDK-LC

COOPERATING UNITS (if any): V. Labroo (LC, NIDDK); J. Daly, C.R. Creveling, F. Gusovsky (LBC, NIDDK); MChanning, D. Kieseewetter, R. Finn (CC, Dept. of Nuclear Med.); D.S. Goldstein, G. Eisenhofer (HE, NHLBI); I.J. Kopin (DIR, NINCDS); M. Linnoila (NIAAA); C.C. Chieuh (NIMH); S. Reppert (Harvard Med. Sch.); G. Rudnik (Yale Univ.); R.S. Phillips (Univ. Georgia)

## LAB/BRANCH

Laboratory of Chemistry

## SECTION

Section on Drug Receptor Interactions

## INSTITUTE AND LOCATION

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## TOTAL MAN-YEARS:

5.8

## PROFESSIONAL:

5.8

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unproduced type. Do not exceed the space provided.)

Biogenic amines play key roles in neurotransmission, metabolism and in control of various physiological processes. Using a variety of synthetic methodologies, including novel procedures developed by us, we have prepared a series of biogenic amines with fluorine substituted at various ring-positions. By virtue of its very small size and high electronegativity, fluorine is a very favorable replacement for hydrogen in these analogs. The biological properties and usefulness of these ring-fluorinated biogenic amines have proved to be extremely rewarding and continue to find applications in a multitude of studies, including research on the mechanisms of transport, storage, release, metabolism, and modes of action of these amines. Of particular significance was the discovery that 6-fluoronorepinephrine is a selective alpha-adrenergic agonist and 2-fluoronorepinephrine is a selective beta-adrenergic agonist. Mechanisms considered to explain these results include: 1) a direct effect of the C-F bond on agonist-receptor interaction or 2) an indirect effect of the C-F bond on the conformation of the ethanolamine side-chain. The results of testing of new analogs synthesized to probe these mechanisms indicate that electronic effects may be more important than conformational factors. Fluorinated analogs are useful biological tracers. For example,  $^{18}\text{F}$ -labeled 6-fluorodopamine, the biological precursor to 6-fluoronorepinephrine, has been found to be an excellent scanning agent for peripheral noradrenergic innervation.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 19607-07 LC

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chemistry, Biochemistry and Pharmacology of Bioindole Analogs

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Louis A. Cohen Chief, Section on Biochem. Mech. NIDDK-LC

Other: Rita Labroo Special Volunteer GWU

## COOPERATING UNITS (if any)

Edith Miles, LBP, NIDDK; Robert Phillips, University of Georgia; Peter Kador, LMOD, NEI; Hiroshi Kimoto, Nagoya, Japan; Walter Dürckheimer, Frankfurt, FRG; Shelly Avramovici, Jerusalem, Israel

## LAB/BRANCH

Laboratory of Chemistry

## SECTION

Section on Biochemical Mechanisms

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.3

## PROFESSIONAL:

0.3

## OTHER:

## CHECK APPROPRIATE BOXES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Studies on the mechanisms of certain tryptophan reactions suggest that the indolenine tautomer should be the true substrate for some tryptophan-metabolizing enzymes. The first conclusive support for this concept is found in our demonstration that 2,3-dihydro-L-tryptophan and oxindolyl-L-alanine, analogs of the indolenine tautomer of tryptophan (tetrahedral carbon at C-3), are potent competitive inhibitors of tryptophan synthase and tryptophanase. Furthermore, the two enzymes show opposing specificity for the C-3 diastereoisomers of 2,3-dihydro-L-tryptophan, suggesting that these enzymes catalyze their reactions via enantiomeric indolenine intermediates. Dioxindolyl-L-alanine (3R) is also an inhibitor of tryptophanase and matches the stereochemistry of (3R)-2,3-dihydro-L-tryptophan, which inhibits the same enzyme. 3-Azido-oxindolyl-L-alanine, a potential photoaffinity label for tryptophanase, has been prepared.

Inhibition of the enzyme aldose reductase represents a new pharmacological approach toward the treatment of late-onset diabetic complications. These complications affect the eye, kidney, nervous system and circulation; they are thought to result from the hyperosmotic effects of high concentrations of sorbitol, in turn resulting from the reduction of the excess glucose symptomatic of diabetes. Our methods for the synthesis of inhibitors of tryptophan-metabolizing enzymes involve spirolactone intermediates which are fairly similar in overall structure to compounds now in clinical trials as aldose reductase inhibitors.

The first series of compounds tested show the spirolactones to be active only at concentrations 100 times those of commercial inhibitors; however, the hydroxy-acids are ten times more active than the lactones. Replacement of the hydroxyl group by halogen and azido provides compounds which may act as affinity and photoaffinity labels. These chiral nonamino acids have been successfully resolved by chymotryptic hydrolysis of their esters.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 19608-06 LC

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Functionalized Congeners of Bioactive Compounds

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: K. Jacobson Research Chemist NIDDK-LC

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COOPERATING UNITS (if any) J. Daly, NIDDK-LBC; J. Baumgold, NINCDS; B. Madras, Harvard University; J. Neumeyer, Research Biochem., Inc.; K. Rice, NIDDK-LMC; A. Jacobson, NIDDK-LMC; A. Lipkowski, Warsaw University

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1.7

## PROFESSIONAL:

1.3

## OTHER:

0.4

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Recent work in our laboratory and in others has demonstrated that certain drugs may be attached to well-defined "carrier" molecules and still retain the ability to bind to the receptor site and effect biological activity. This synthetic strategy for the attachment of drugs to carriers is termed the "functionalized congener" approach. The "carrier" molecule may be many times larger than the parent drug; indeed there is practically no maximum size limitation for a fully potent analog. Unlike the prodrug approach or the immobilization of drugs for slow release, the "functionalized congener" approach is designed to produce analogs for which no metabolic cleavage step is necessary for activation. Moreover, the attachment of the drug to a "carrier" such as a peptide may result in the enhanced affinity at an extracellular receptor site and an improvement in the pharmacological profile of the parent drug.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 19610-02 LC

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Prosthetic Groups for Radiolabeling of Functionalized Drugs and Peptides

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: K. Jacobson Research chemist NIDDK-LC

Other: K. Kirk Research Chemist NIDDK-LC  
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## LAB/BRANCH

Laboratory of Chemistry

## SECTION

Section on Drug Receptor Interactions

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.7

## PROFESSIONAL:

0.7

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The use of radioisotopes to label organic compounds for use in diagnostic nuclear medicine is well documented in the literature. It has been found that certain radiolabeled compounds will localize in the brain, heart, or in other target organ or tissues to a sufficient level to allow for imaging thereof. There has been increasing interest in finding compounds which will more effectively cross the blood-brain barrier, thus facilitating more efficacious imaging of the brain.

Binding sites for certain drugs in an animal or organ may be localized as a result of the synthesis of high specific activity radiolabeled analogs which have high affinity for that binding site. Prosthetic groups may be attached to a drug or other receptor ligand for the purpose of efficient and selective chemical capture of a particular radioisotope. Having developed functionalized congeners of theophylline and other drugs acting at adenosine receptors, we are now developing prosthetic groups for radioisotopes such as  $^{18}\text{F}$ ,  $^{123}\text{I}$ , and  $^{125}\text{I}$ , to be coupled to these functionalized drug molecules. The prosthetic groups contain amino or carboxylic groups which are to be condensed covalently to functionalized drugs to give conjugates of high affinity at a particular receptor.

Positron emission tomography (PET) has been used for imaging receptors in the brain and other organs. A prosthetic group for chemical capture of  $^{18}\text{F}$  requires rapid and efficient reaction and purification, since the half-life is only 110 minutes.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 19611-02 LC

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Development of Drugs Acting at Adenosine Receptors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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	S. Barone	Special Volunteer	NIDDK-LC
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COOPERATING UNITS (if any) J. Daly (NIDDK), B. Fredholm (Karolinska Inst.), J. Neumeyer (Res. Biochem., Inc.), P. Churchill (Wayne State), G. Stiles (Duke Univ.), P. Marangos (NIMH), M. Williams (CIBA-GEIGY), G. Evoniuk (NIDDK-LN), P. Morgan (NIMH), L. Wang (Univ. of Alberta)

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Section on Drug Receptor Interactions

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## TOTAL MAN-YEARS:

1.1

## PROFESSIONAL:

0.7

## OTHER:

0.4

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

The extracellular adenosine receptor has a modulatory role in the nervous, circulatory, endocrine, and immunological systems. The prospect of harnessing these effects specifically for therapeutic purposes is attractive.

A functionalized congener approach to drug design has been applied to the adenosine receptor to produce analogs of agonists and antagonists which have promise as therapeutic agents and as receptor probes. In the antagonist series new analogs which combine potency, water solubility, and A<sub>1</sub>-adenosine receptor selectivity in the same compound are now being evaluated in in vivo testing.

ANNUAL REPORT OF THE LABORATORY OF CELL BIOLOGY AND GENETICS  
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The Laboratory of Cell Biology and Genetics carries on a broad program of investigation into exocrine and endocrine secretion and the molecular events regulating these processes. Four specific tissues have been studied: chromaffin cells, which secrete adrenaline, ATP and endogenous opiates; pancreatic beta cells, which secrete insulin; nerve terminals which secrete transmitters and mucin-secreting colon carcinoma cells which secrete mucins. The following studies have been performed, in pursuit of these goals:

I. EXOCYTOSIS FROM CHROMAFFIN CELLS.

1. Electron Probe Microanalysis of the Subcellular Compartments of Bovine Adrenal Chromaffin Cells:  
COMPARISON OF CHROMAFFIN GRANULES IN SITU AND IN VITRO

The elemental and water content of cultured bovine adrenal chromaffin cells and their secretory chromaffin granules have been measured and compared with isolated chromaffin granules using quick freezing, ultracryomicrotomy, and electron microprobe analysis methods. In units of millimole/kilogram dry weight ( $\pm$  S.E.) granules *in situ* contained: P,  $523 \pm 32$ ;  $K^+$ ,  $124 \pm 9$ ; S,  $82 \pm 3$ ;  $Cl^-$ ,  $74 \pm 9$ ;  $Ca^{2+}$ ,  $13 \pm 2$ ;  $Mg^{2+}$ ,  $6 \pm 2$ ; and  $Na^+$ ,  $2 \pm 2$ . Following routine isolation in isotonic sucrose buffer, granule K and  $Cl^-$  had decreased while granule  $Na^+$  increased.  $Cl^-$  exhibited a consistent decrease to 35-40 mmol/kg dry weight. Granule  $Na^+$  and  $K^+$  concentrations ranged from 43 to 12 mmol/kg and 28 to 60 mmol/kg dry weight, respectively, depending on the  $Na^+$  and  $K^+$  content of the buffer. Despite the redistribution of monovalent ions, granule  $Ca^{2+}$ , granule P, being in the form of ATP, and granule S, being in the form of protein, were not significantly changed. The stability of these elements is consistent with the existence of a stable storage complex for  $Ca^{2+}$ , ATP, and protein.

Using the granule as an internal standard with a water content of 66%, the water contents of external space, nucleus, cytoplasm, and mitochondria were estimated to be 89, 88, 82, and 70%, respectively. Wet weight concentrations for each element were calculated for granules and cytoplasm from which the transgranular concentration gradients for  $K^+$ ,  $Cl^-$ , and  $Na^+$  were determined.  $Cl^-$ , a permeant anion, was 2-fold higher in the granule than in the cytoplasm while  $K^+$ , a slightly permeant cation, had an opposite distribution ratio slightly less than two.

Together, the  $K^+$  and  $Cl^-$  data suggest the presence of an inside-positive granule membrane potential of approximately 10-16 mV. The surprising lack of  $Na^+$  from the granule matrix suggests a high inward gradient for  $Na^+$  even though the  $Na^+$  content of chromaffin cell cytoplasm is low at 5 mmol/kg water. The lack of an outward  $Na^+$  gradient is important in that it indicates that the previously described electroneutral  $Na^+-Ca^{2+}$  exchange system, by which isolated granules accumulate  $Ca^{2+}$ , does not operate in mature granules *in situ*. Consequently, if chromaffin granules regulate internal calcium during stimulus secretion coupling, a mechanism other than  $Na^+-Ca^{2+}$  exchange is necessary.

## 2. Exocytosis of Bovine Chromaffin Granules in Ficoll Captured by Rapid Freezing.

Exocytosis of cultured bovine adrenal chromaffin cells was examined in a balanced salt solution containing Ficoll using rapid freezing followed by freeze-substitution. In solution without Ficoll, exposure to  $Ba^{2+}$  produced many exocytotic lumina between the cells: in most cases, these lumina were large and empty. When chromaffin cells were exposed to  $Ba^{2+}$  in Ficoll, it was possible to observe a small pore between the plasmalemma and each granule. The granule retained the dense contents when the pore was approximately 20 nm in diameter, and became empty when the pore was widened in 14% Ficoll. The addition of Ficoll made it easy to catch the events occurring during exocytosis of individual bovine chromaffin granules.

## 3. Quantitative Electron Energy Loss Spectroscopy To Determine Water Content in Frozen Thin Sections of Chromaffin Cells.

The potential for applying electron energy loss spectroscopy (EELS) to biological problems is becoming better realized with recent developments in instrumentation, spectrometer design, parallel detection and elemental mapping. We have demonstrated quantitation by means of the spectrum from DNA which gives an elemental ratio for N:P close to the expected value. A range of biologically important elements that can be usefully analyzed by EELS includes C, Na, K, C, S, Ca, Mg, P, N and others of higher atomic weight. Detection limits and the effects of radiation damage have been studied using spectra from the protein, insulin, and from the fluorinated amino-acid, histidine. Calcium detectability under optimum conditions may be as low as 1 mmol/kg dry weight. We have applied EELS to analysis of cryosectioned adrenomedullary (chromaffin) cells in order to help determine the composition of the secretory granule. Water content can be determined from the amount of inelastic scattering as measured by the low-loss spectrum. The nitrogen/phosphorus ratio can be measured to provide information about the relative concentrations of ATP, chromogranin, and catecholamines. Quantitative EELS

elemental maps are obtained in the STEM mode from chromaffin cells in order to measure the distribution of light elements.

4. Ba<sup>2+</sup>-Induced ATP Release From Adrenal Medullary Chromaffin Cells is Mediated by Ba<sup>2+</sup> Entry Through Both Voltage- and Receptor-Gated Ca<sup>2+</sup> Channels.

We have measured on-line the exocytotic of ATP from adrenal medullary chromaffin cells induced by Ba<sup>2+</sup> using a luciferin/luciferase assay. We have found that Ba<sup>2+</sup>-induced ATP release requires the entry of Ba<sup>2+</sup> through either voltage- or receptor-gated Ca<sup>2+</sup> channels. This conclusion is based on the observations that short preincubations with low concentrations of either nicotine or K<sup>+</sup> greatly enhance Ba<sup>2+</sup>-induced ATP release and that this augmentation can be blocked with the nicotinic receptor antagonist, hexamethonium, and the Ca<sup>2+</sup> antagonist, nifedipine, respectively. Moreover, both nicotine and K<sup>+</sup> stimulate <sup>133</sup>Ba<sup>2+</sup> uptake, which in the case of K<sup>+</sup> is inhibited by nifedipine. These results support the hypothesis that the cellular events leading to Ba<sup>2+</sup>-induced secretion coincide at least in part with the events leading to Ca<sup>2+</sup>-dependent exocytosis.

5. Barium Ions Enter Chromaffin Cells via Voltage-dependent Calcium Channels and Induce Secretion by a Mechanism Independent of Calcium.

Barium ions enter chromaffin cells via voltage-sensitive calcium channels, although the intracellular site of barium action is distinct from that of calcium. The entry of barium primarily through voltage-sensitive channels was indicated by experiments showing inhibition of <sup>133</sup>Ba<sup>2+</sup> uptake by blockers of voltage-dependent calcium channels. In addition, <sup>133</sup>Ba<sup>2+</sup> uptake was stimulated by 50 mM KCl but not by nicotine. Furthermore, <sup>133</sup>Ba<sup>2+</sup> uptake was inhibited by hyperosmolarity, which specifically blocks the voltage-sensitive calcium channel but not the receptor-associated calcium channel. These conclusions from studies on barium uptake were also borne out by experiments measuring catecholamine secretion. Thus, blockers of voltage-dependent calcium channels which inhibited barium uptake also inhibited barium-induced catecholamine secretion. In other experiments, simultaneous stimulation with nicotine and barium in the presence of calcium evoked coincident and additive catecholamine secretion. By contrast, when 50 mM KCl was substituted for nicotine in the same experimental design, barium ions inhibited potassium-induced catecholamine secretion at low calcium concentrations. Only at high calcium concentrations were barium-induced and potassium-induced secretion additive. These data also indicate that barium and calcium compete at the voltage-sensitive pathway. Furthermore, these additivity data suggest that once inside the cell, barium and calcium have two distinct mechanisms of action.

As predicted by this hypothesis, in digitonin-permeabilized chromaffin cells either calcium or barium stimulated catecholamine release, and in the presence of both cations catecholamine secretion was equivalent to the sum of secretion with either cation alone. Additional support of this concept comes from experiments showing that while calcium-mediated catecholamine secretion is sensitive to trifluoperazine and imipramine, barium-mediated secretion is not. Taken together, all these data indicate that there are two distinct intracellular sites of action for barium and calcium. In contrast to catecholamine secretion, non-exocytotic ascorbic acid secretion was induced by nicotine and potassium in the presence of calcium, but not by barium alone. These data provide additional evidence that barium acts by a different mechanism than calcium, in still another secretory system in chromaffin cells.

6. Interaction of protein kinase C with chromaffin granule membranes: effects of  $\text{Ca}^{2+}$ , phorbol esters and temperature reveal differences in the properties of the association and dissociation events.

Interaction of protein kinase C with chromaffin granule membranes has been studied as a means of investigating the translocation of protein kinase C from cytosol to intracellular membrane surfaces, which is believed to occur during secretion. Protein kinase C in an adrenal medullary soluble fraction was found to bind reversibly to granule membranes in a  $\text{Ca}^{2+}$ -dependent fashion. Association and dissociation events were sensitive to  $\text{Ca}^{2+}$  concentrations in the low micromolar range, and the  $\text{Ca}^{2+}$  sensitivity of both processes was increased when the membranes had been preincubated with the protein kinase C-activating phorbol ester, 4 $\beta$ -phorbol 12-myristate 13-acetate (TPA). Binding of protein kinase C to granule membranes occurred at 0 and 37° C, irrespective of whether the membranes had been preincubated with TPA. However, dissociation of protein kinase C from granule membranes that had been preincubated with TPA occurred only at 37° C and not at 0° C, even though dissociation of the enzyme from membranes which had not been preincubated with TPA would occur at both 37 and 0° C. These effects of TPA were not reproduced by 4 $\alpha$ -phorbol 12,13-didecanoate (4 $\alpha$ PD), a phorbol ester which does not activate protein kinase C. Soluble protein kinase C activity also associated with chromaffin granules in a  $\text{Ca}^{2+}$ -dependent manner in an adrenal medullary homogenate, indicating that granules can compete with other intracellular membranes for the binding of protein kinase C. Results obtained with this model system differ from other systems where the interaction of protein kinase C with plasma membranes has been studied and have general implications for studies performed on the translocation of protein kinase C in intact cells and for the role of protein kinase C in stimulus-secretion coupling in the chromaffin cell.

7. Ascorbic Acid and Mg-ATP Co-regulate Dopamine  $\beta$ -Monooxygenase Activity in Intact Chromaffin Granules.

Ascorbic acid and Mg-ATP were found to regulate norepinephrine biosynthesis in intact secretory vesicles synergistically and specifically, using the model system of isolated bovine chromaffin granules. Dopamine uptake into chromaffin granules was shown to be unrelated to the presence of Mg-ATP and ascorbic acid at external dopamine concentrations of 7.5 and 10 mM. Under these conditions of dopamine uptake, norepinephrine biosynthesis was enhanced 5-6 fold by Mg-ATP and ascorbic acid compared to control experiments with dopamine only. Furthermore, norepinephrine formation was enhanced approximately 3-fold by ascorbic acid and Mg-ATP together compared to norepinephrine formation in granules incubated with either substance alone. The action of Mg-ATP and ascorbic acid together was synergistic and independent of dopamine content of chromaffin granules as well as of dopamine uptake. The apparent  $K_m$  of norepinephrine formation for external ascorbic acid was 376  $\mu M$ , consistent with the larger amounts of cytosolic ascorbic acid and ATP that are available to chromaffin granules. Other physiologic reducing agents were not able to increase norepinephrine biosynthesis in the presence or absence of Mg-ATP. In addition, maximum enhancement of norepinephrine biosynthesis occurred only with the nucleotide ATP and the cation magnesium. The mechanism of the effect of ascorbic acid and Mg-ATP on norepinephrine biosynthesis was investigated and appeared to be independent of a positive membrane potential. The effect was also not mediated by direct action of ADP, ATP, or magnesium on the activity of soluble or particulate dopamine  $\beta$ -monooxygenase. These data indicate that Mg-ATP and ascorbic acid specifically and synergistically co-regulate dopamine  $\beta$ -monooxygenase activity in intact chromaffin granules, independent of substrate uptake. Although the mechanism is not known, the data are consistent with the possibility that the chromaffin granule ATPase mediated these effects.

8. Role of Intracellular pH in Secretion from Adrenal Medulla Chromaffin Cells.

The role of intracellular pH in stimulus-secretion coupling was investigated in cultured bovine adrenal medullary chromaffin cells.  $NH_4Cl$  (1-25 mM) did not affect basal catecholamine or ATP release but markedly inhibited nicotine- or high  $K^+$ -induced release by up to 60%. The inhibition had a rapid onset (<1 min) and was maximal at about 5 mM  $NH_4Cl$ . The effect of  $NH_4Cl$  was largely sustained over 20 min and was reversed upon  $NH_4Cl$  removal. Sodium propionate did not affect secretion but partially reversed the inhibition by  $NH_4Cl$  in a concentration-dependent manner. Methylamine (10 mM) produced a similar, but slower, inhibition than  $NH_4Cl$ . Monensin (1-10  $\mu M$ ) inhibited catecholamine secretion

by 30-60%, and its effect was reduced in the presence of  $\text{NH}_4\text{Cl}$ . Using the fluorescent  $\text{Ca}^{2+}$  probe Fura-2, we found that the increase of  $[\text{Ca}^{2+}]_i$  following stimulation was not altered by concentrations of  $\text{NH}_4\text{Cl}$  which inhibited secretion maximally. Measurement of cytosolic pH ( $\text{pH}_i$ ) with the fluorescent probe 2',7'-bis-carboxyethyl-5(6)-carboxyfluorescein (BCECF) revealed an alkalinization by  $\text{NH}_4\text{Cl}$  (2.5 mM) of 0.1-0.23 pH units and acidification by sodium propionate (10-20 mM) of 0.2-0.25 pH units, with intermediate combined effects. Monensin (1  $\mu\text{M}$ ) caused a cytosolic acidification of 0.26 pH units. All  $\text{pH}_i$  changes were partly recovered in 15 min. Fluorescence quenching measurements using the weakly basic fluorescent probe acridine orange indicated the accumulation of the probe into acidic compartments, presumably the chromaffin granules, which was strongly reduced by both  $\text{NH}_4\text{Cl}$  and monensin. From these findings we conclude that the pH of the chromaffin granule modulates secretion by affecting some step in the secretory process unrelated to the rise in  $[\text{Ca}^{2+}]_i$ .

9. Evidence that Calcium Ions Enter Chromaffin Cells Through A Voltage-Dependent pathway Which is Insensitive to Dihydropyridines and W-Conotoxin.

The increase in intracellular free calcium concentration ( $[\text{Ca}^{2+}]_i$ ) that follows depolarization of the chromaffin cell is thought to play an important role in the exocytotic release of catecholamines. We have studied the modulation of  $[\text{Ca}^{2+}]_i$  by membrane potential in bovine adrenal medulla chromaffin cells by monitoring the fluorescence of cells loaded with the  $\text{Ca}^{2+}$  indicator FURA II. Following stimulation with high  $\text{K}^+$  concentrations (10-65 mM),  $[\text{Ca}^{2+}]_i$  first rapidly increased to a peak value and then decreased to a "plateau" level with a much slower time course. The relationship between the rapid  $[\text{Ca}^{2+}]_i$  increase ( $\Delta [\text{Ca}^{2+}]_i$ ) and the calculated membrane potential could be well described by the Boltzmann distribution function for two state transitions (half-maximal activation at -23 mV).  $\Delta [\text{Ca}^{2+}]_i$  was abolished by EGTA and increased with  $[\text{Ca}^{2+}]_o$  (0.1-2 mM) in dose-dependent fashion. Preincubating the cells with the Ca channel antagonist  $\text{La}^{3+}$  (4-40  $\mu\text{M}$ ) suppressed  $\Delta [\text{Ca}^{2+}]_i$  when added after  $\text{K}^+$  stimulation. While the dihydropyridine (DHP) agonist +202-791 increased  $[\text{Ca}^{2+}]_i$  in the presence of 30 mM  $\text{K}^+$ , thus indicating the presence of a DHP receptor in these cells, DHP antagonists (1-5  $\mu\text{M}$  nifedipine or nitrendipine) reduced  $\Delta [\text{Ca}^{2+}]_i$  by only ca. 20%, regardless of the membrane potential in the range -25/-5 mV. W-conotoxin (0.1-1  $\mu\text{M}$ ) did not reduce  $\Delta [\text{Ca}^{2+}]_i$ . We propose that depolarization activates a DHP-insensitive Ca channel in chromaffin cells, and the  $\text{Ca}^{2+}$

influx brought about by depolarization generates  $\text{Ca}^{2+}$  release from intracellular stores.

10. Temporal Relationship Between Calcium Entry and Secretory Activity in a Single Adrenal Chromaffin Cell in Culture.

The patch clamp technique was used in the whole cell recording mode to measure membrane currents and membrane fusion in adrenal chromaffin cells in culture. To elicit calcium entry, three to five brief ( $< 50$  msec) voltage clamp pulses from  $-80$  to  $0$  mV were applied and the ensuing changes in cell membrane capacity  $C_m$  thought to be associated with membrane fusion were recorded.  $C_m$  was measured as  $Q/V$  where the charge  $Q$  was estimated from the fast current transients in response to brief ( $< 50$  msec) hyperpolarizing pulses. Application of a series of pulses which did not elicit calcium currents (i.e. from  $-80$  to  $-110$  mV), induced no measurable changes in  $C_m$ . In contrast, application of a few depolarizing pulses (i.e. from  $-80$  to  $0$  mV) which induced measurable calcium currents to the same cell, evoked a marked increase in  $C_m$ . Immediately after calcium entry (from  $0.3$  to  $0.6$  fmol/cell),  $C_m$  rose from its average resting value of  $4.5$  pF to a maximum value of  $7.2$  pF with a single time constant in the range from  $15$  to  $70$  sec. This increase occurred only if calcium were present in the external medium. The increase in  $C_m$  due to the fusion of a single secretory vesicle into the plasma membrane, estimated as  $1$  fF (area of the granule  $\times$  specific membrane capacity), could not be resolved with the present technique. However, from the extent of the capacity increase (about  $2$  pF) we estimate that the calcium entry during the few pulses caused nearly  $2,000$  secretory vesicles to fuse with the plasma membrane of the chromaffin cell. Assuming  $10,000$  granules/cell, the size of the component of the secretion which is strictly dependent on extracellular calcium amounts to  $20\%$  of the granular pool. Since the total calcium entry amounted to  $0.4$  fmol/cell, we estimate that ca.  $120,000$  calcium ions per granule are needed for the fusion event to occur.

11. Ionic Composition of the Medium Regulates Osmotic Fragility of Secretory Granules.

Osmotic gradients across secretory granule membranes are believed to be important in the regulation of exocytosis and as a driving force for this process. This view was proposed in the chemiosmotic hypothesis for secretion. As the chromaffin cell model is among the best studied in terms of chemiosmotic processes, we studied mechanisms regulating this process in chromaffin granules. It has been shown that granules in situ and in vitro differ with respect to their osmotic fragility. Understanding why this occurs is central to understanding the energetics of secretion. We have found that if granules are equilibrated in an isoosmotic medium reflecting measured concentrations of  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^+$ ,  $\text{Mg}^{++}$  and  $\text{Ca}^{++}$  in the cytosol, the

osmotic fragility of these granules in fact closely approximates that found in intact cells.

Osmotic fragility experiments of isolated chromaffin granules in various osmolar concentration of sucrose showed that the granules were stable in isoosmotic sucrose (340 mOsm). As the osmolarity of sucrose was decreased, the osmotic fragility of the granules significantly increased. Approximately 50% of the granules lysed in moderately hypoosmotic sucrose (240 mOsm). When, however, the osmotic fragility of granules was tested in various dilutions of an ionic solution representing the ionic milieu of the cytosol, the granules were surprisingly stable; 50% of the granules only lysed when the media was very hypoosmotic (34 mOsm). These values parallel osmotic fragility in intact cells. The high osmotic fragility of granules isolated in isoosmotic sucrose was reversed by transferring the granules to the above ionic media. This indicates that granule damage during the isolation procedure was not responsible for the high osmotic fragility. When the  $K^+$  content of the salt media was replaced by sucrose, the osmotic fragility curve shifted in a direction indicating an enhanced fragility. Substitution of  $K^+$  with various monovalent cations showed  $K^+ \geq Na^+ \geq Rb^+ > Cs^+ >> Li^+$ . These results indicate that osmotic fragility depends on the ionic composition of the granule environment. Further, no difference was observed in the osmotic fragility of granule ghosts, loaded with isoosmotic  $K^+$ -glutamate, when suspended in various osmolar solutions of sucrose or  $K^+$ -glutamate. These results are indicative of the importance of the granule core in this stabilization process. In addition, the results suggest that varying the ionic composition of the medium can result in the generation of an osmotic gradient across the granule membrane, without changes in the osmolarity of the external medium or transport into the granule of osmotically active ions.

These data indicate that the ionic composition of the suspension medium plays a crucial role in regulating the osmotic fragility of secretory granules. The possible involvement of this process in vivo during secretory events is currently being pursued.

## II. MECHANISM OF SYNEXIN ACTION TO PROMOTE MEMBRANE FUSION.

1.  $Ca^{2+}$ -activated synexin forms highly selective, voltage-gated  $Ca^{2+}$  channels in phosphatidylserine bilayer membranes.

Synexin, a cytosolic protein which mediates  $Ca^{2+}$ -dependent membrane fusion, was incorporated into acidic phospholipid bilayers, formed at the tip of a patch pipet. The pipet was filled with a high- $Ca^{2+}$  solution (50 mM) and immersed in a chamber containing a low- $Ca^{2+}$  solution (1 mM). Brief exposures of the bilayer to synexin increased the capacitance of the bilayer by a

factor of 10 and decreased the membrane resistance by a factor of 20. Reduction of  $\text{Ca}^{2+}$  in the chamber to 1  $\mu\text{M}$  caused an abrupt increase in the current required to hold the pipet potential at 0 mV. Under certain conditions channel events could be detected, often occurring in bursts. Consistently, open-time histograms were found to be voltage-dependent and to exhibit one time constant in the time range examined here. The slope conductance for the synexin channel was estimated as  $10.2 \pm 2.1$  pS for the large  $\text{Ca}^{2+}$  gradient with low chamber  $\text{Ca}^{2+}$ . However, for symmetrical, low- $\text{Cl}^-$  solutions containing 25 mM  $\text{Ca}^{2+}$  the conductance was  $26.5 \pm 5.2$  pS. Ion-replacement studies showed the synexin channel to much prefer  $\text{Ca}^{2+}$  over  $\text{Ba}^{2+}$  or  $\text{Mg}^{2+}$ .  $\text{Cd}^{2+}$ , a potent blocker of other voltage-gated  $\text{Ca}^{2+}$  channels at 100  $\mu\text{M}$ , blocked synexin channels only at very high concentrations ( $\geq 10$  mM). Similarly, nifedipine, an inhibitor of the nonactivating  $\text{Ca}^{2+}$  channel, was effective only at extremely high concentrations ( $> 300$   $\mu\text{M}$ ). The high selectivity for  $\text{Ca}^{2+}$  and the lack of response of the channel to various drugs known to block  $\text{Ca}^{2+}$  channels thus distinguish the synexin channel from other types of  $\text{Ca}^{2+}$  channels hitherto reported.

## 2. Derived Amino Acid Sequence of Human Synexin: A Protein with Calcium Channel Activity and Similarities with Endonexin II-like Proteins.

Nucleotide probes based on amino acid sequence of tryptic fragments were used to identify clones from human lung and liver libraries. These cDNAs plus regions of other ones from of B-cell, retina and fibroblast libraries were sequenced. All of the human synexin peptides were identical to the predicted protein sequence, while only minor differences were observed with the bovine samples. We show that the amino acid sequence from the synexin cDNA clones reveals strong similarity with endonexin-like proteins (porcine protein II, P2; endonexin II, E2; calelectrin, C; lipocortin I, L1; and calpactin I, C1). The N-terminal region of the endonexin-like proteins are different and tend to be short, except for synexin which is longer (163 amino acids versus 16-46) and more hydrophobic. However, the synexin sequence in the C-terminal region contains four conserved repeats of about 70 residues each and reveals 45+5% identity (except repeat 7 of calelectrin) with the other proteins. Each of these proteins bind to acidic phospholipids in a calcium dependent manner and this domain(s) is likely to reside in this region. We derived a 2-D model from a hydrophobicity plot of the conserved region. The four repeat regions were used to form sides of an imaginary box with the hydrophobic and hydrophilic stretches of amino acids being placed outside or inside, respectively, of the lines forming a box. The hydrophobic character of the exterior of the box could interact with the membranes and the hydrophilic interior with a net charge of -7 could be responsible for the observed calcium channel activity.

### 3. Alternative Splicing and Polyadenylation of Human Synexin mRNA.

Synexin is a calcium dependent membrane binding protein that aggregates adrenal chromaffin granules and acts as a voltage-dependent calcium channel in artificial bilayers. Human synexin cDNA clones have been used to analyze messenger RNA for synexin. Northern blot analysis of human liver and adrenal medulla mRNA revealed the existence of two distinct synexin bands of 1.95 Kb and 2.4 Kb. The possibility of alternative splicing was examined by isolating and sequencing several human synexin cDNA clones. Two of the clones analysed contained a longer 3' untranslated region (336 bp) generated by the selection of an alternative polyadenylation site. We have also isolated a cDNA clone containing a presumed exon (66 bp) located at the 5' end region. The deduced aminoacid sequence of this exon introduces 3 acidic aminoacids to an otherwise highly hydrophobic, unique N-terminal domain. The prevalence of these transcripts is being analysed by screening specific regions of human synexin mRNA using the polymerase chain reaction technique and detecting the amplified products with oligonucleotide probes. The functional role of these putative alternative splicing is being investigated.

### 4. Hydrophobic Bridge Hypothesis for Synexin-Driven, Calcium-Dependent Membrane fusion.

Membranes of secretory vesicles fuse with each other and with plasma membranes during exocytosis in many different cell types. The probable role of calcium in the process is now widely accepted, and it is possible that at least one cytosolic mediator of calcium action is synexin is a 47000 Mr calcium-binding protein, initially discovered in the bovine adrenal medulla, which binds to granule membranes and to inner aspects of chromaffin cell plasma membranes. Synexin causes chromaffin granules to aggregate, and such aggregates can be caused to fuse in the additional presence of arachidonic acid. Synexin also mediates the direct fusion of liposomes and chromaffin granule ghosts. To understand better the mechanisms of membrane fusion promoted by synexin we have attempted to define the primary sequence of the protein. Our initial efforts were directed towards purification of bovine synexin in sufficient amounts to allow us to sequence tryptic peptides. However, as the project progressed we also directed our attention to human synexin, preparing peptides from this protein as well. From analysis of bovine peptides we learned that the synexin molecule might be closely related to a class of proteins including lipocortin I, calpactin (p36), endonexin II, protein II and calelectrin 67K. Complete analysis of a human synexin cDNA clone revealed strong homology with bovine synexin. The analysis also showed that synexin contained a unique, long, highly hydrophobic N-terminal leader sequence followed by a characteristic four-fold repeat homologous with those found in other members of the synexin gene family. The highly hydrophobic

character of synexin seems consistent with information previously obtained that synexin is able to insert directly into the interior of bilayers prepared not only from purified phosphatidylserine but also from biological membranes. The evidence for such insertions is a dramatic increase in the capacitance of the membrane, formed at the tip of a patch pipette, when calcium-activated synexin is applied to the bilayer. Additional evidence is the fact that synexin also forms calcium-selective channels when the protein is applied to the cytosolic aspect of the plasmalemma when that side is also exposed to calcium at sub-millimolar concentrations. Thus, the synexin molecule not only enters the membrane, but also spans it. From these and other data we have developed the concept that the fusion process may involve synexin forming a 'hydrophobic bridge' between two fusing membranes. Lipid movement across this bridge may then be the material basis for final fusion.

### III. MECHANISMS REGULATING INSULIN SECRETION FROM RODENT AND HUMAN BETA CELLS FROM ISLETS OF LANSERHANS.

#### 1. Three K Channels in Human Pancreatic B-Cells Identical to Those in Rat B-Cells.

Several K channels have been described in rat and mouse B-cells, three have been well characterized in several laboratories, and two are known to be sensitive to glucose in cell-attached patches of membrane. We compared the properties of the K channels in B-cells from the pancreas of a normal human transplant donor (NDRI) with those obtained from rat pancreases. Islet were hand picked after collagenase digestion; B-cells were isolated using EDTA and dispase and cultured in CMRL 1066 medium (Gibco) with 5.6 mM glucose for 1 to 15 days. Standard patch clamp methods were used. K(Ca) channels had a conductance of 95 pS for outward currents (135 mM Na in pipet; 140 K in bath) and the open probability was reduced by addition of 20 mM glucose. K(ATP) channels showed rectification and had a conductance of 55 pS for inward currents (140 K in pipet) and 18 pS for outward currents (135 Na in pipet) and were closed by 20 mM glucose in cell attached patches and by ATP in excised patches. The small 8 or 9 pS K channel was occasionally seen, but did not appear to be sensitive to glucose. These results indicate that K channels in human pancreatic B-cells are identical to those in rat pancreatic B-cells and indicate that the rat islet model may be used to study normal human islet behaviour. Furthermore, these results confirm the involvement of two K channels in the process of glucose sensing in pancreatic B-cells.

#### 2. Contrast Between Acute Phase and Secondary Phase Insulin Secretion Dependence upon Oscillatory Membrane Potential for Glucose Sensitivity in Rodent Islets.

Glucose depolarizes the B-cell membrane and above 8 mM, induces regular oscillations between a silent phase and an active phase. B-cells exhibit a graded increase in duration of active phase (depolarized and spiking) with increasing glucose. Both the

oscillatory and graded responses have been closely correlated with insulin secretion. The objective of the current work was to study acute and steady state insulin release in response to graded glucose in the presence of a fixed depolarized membrane potential. KCl (25 mM) depolarized the membrane to -25 mV without oscillations or action potentials. Glyburide (4  $\mu$ M) depolarized the membrane to -25 mV without oscillations but with action potentials. No graded electrical glucose response was evident in either condition. We studied acute phase insulin secretion from perfused microdissected mouse islets exposed to 10 minute graded glucose increments from 5.6-33 mM glucose. Secondary phase insulin release at the same glucose concentrations was studied using 90 min. static incubations of collagenase-isolated rat islets. Glyburide and 25 mM KCl induced significant increases in acute phase basal (5.6 mM) insulin release (23% vs 88% increases respectively  $p < .05$ ). In addition, a significant graded glucose evoked insulin secretion comparable to that observed from control islets was elicited in both conditions. The basal long term insulin release was significantly increased by KCl-depolarization; however, there was no graded insulin response to glucose. We conclude that once the cell is depolarized beyond the threshold, acute phase insulin release probably reflects glucose sensitive, or receptor activated, second messenger generation whereas secondary phase glucose sensitive insulin release is membrane potential dependent and reflects the major role of calcium entry through voltage gated calcium channels.

### 3. Effects of Ca-Channel Agonist-Antagonist Enantiomers of Dihydropyridine 202-791 on Insulin Release, Ca Uptake and Electrical Activity in Isolated Pancreatic Islets.

Ca-Channels in the B-cell membrane link the glucose-induced depolarization to insulin release. The + and - forms of the dihydropyridine, 202-791 (Sandoz), which acts as a Ca-channel agonist or antagonist, were studied using collagenase isolated rat islets to measure Ca uptake and insulin release and micro-dissected mouse islets to measure membrane potential. Glucose induced insulin secretion and Ca uptake were inhibited about 50% by the antagonist, (-)202 (10 or 100  $\mu$ M), while K induced insulin release and Ca uptake were inhibited 100% (10  $\mu$ M). Addition of the agonist, (+) 202 (10  $\mu$ M) enhanced insulin release and Ca uptake over basal levels, but failed to further increase the glucose induced insulin release or Ca uptake. The (-) 202 form was a stronger antagonist to the (+) 202 form than was nifedipine. The effects of the (+) and (-) forms of 202-791 on the membrane potential and electrical activity were complex (similar to the effects of BAY K 8644 to inhibit and nifedipine to stimulate) illustrating the close association of K channel activation with Ca currents in the B-cell. In the presence of a K channel blocker, TEA, (+) 202 increased and (-) 202 decreased the duration of the action potentials. The results confirm the existence of a dihydropyridine sensitive Ca channel in B-cells and indicate the presence of another Ca channel which is insensitive to dihydropyridines and is perhaps activated by glucose.

#### 4. Barium Inhibits Ca-Activated K Channels in Pancretic B-Cells.

In mouse pancreatic B-cells, glucose reduces K permeability, depolarizes the membrane, induces Ca action potentials and increases insulin release. Ba mimics these effects. In order to determine which K channels are involved in glucose sensing, we have studied the effects of Ba and its competition with Ca on the K permeability in B-cells from mouse and rat using standard intracellular potential recording and patch clamp techniques. Ba (0.5 - 5 mM) depolarized mouse B-cells, induced spikes and increased the input membrane resistance either in the absence or presence of 2.5 mM Ca. Spikes augmented in duration with increasing [Ba]. In inside-out patches from cultured rat B-cells (high Na in the pipette), 0.1 to 10 mM Ba decreased the open time probability of the Ca-activated K-channel, K (Ca), in the presence of 0.1 mM Ca. Increasing [Ca] from 0.1 to 0.5 mM partially reversed this inhibition. Also, the K(Ca) channel exhibited a sub-state conductance when Ba and Ca were present. In whole cell configuration, Ba inward currents were larger than Ca currents and did not inactivate. Essentially, Ba replaces Ca in the inward current, but competes with it for the membrane depolarization and increased input resistance. These data indicate to us that the K(Ca) channel plays an important role in the control of B-cell membrane potential.

#### 5. Effects of Glucose and Bicarbonate on the PH of the Inter-cellular Space in Mouse Islets of Langerhans.

Mouse islets of Langerhans consist mainly of tightly packed B-cells which secrete insulin in response to glucose; the intercellular space (excluding capillary space) represents less than 1% of the islet volume. B-cells are electrically synchronous, and during the burst pattern of electrical activity, K is accumulated and Ca is depleted in the intercellular space. Glucose sensitivity of the B-cell is controlled by K permeability; two of the K-channels described have been reported to show pH sensitivity. Although glucose metabolism may be expected to acidify the cytosol, and thus transiently acidify the intercellular space, measurements of cytosolic pH in B-cells are conflicting. Single microdissected mouse islets (0.5 mm minimum diameter) were continuously perfused while measuring the intercellular pH with a liquid H-sensitive micro-electrode. The intercellular space at the center of the islet was slightly more acidic than the bath solution; addition of 22 mM glucose induced a slight alkalization. Reducing the NaHCO<sub>3</sub> concentration from 25 to 5 mM and the CO<sub>2</sub> from 5% to 1% decreased the buffering capacity of the solution without changing the pH from 7.4; this decreased the intercellular pH to 6.6 (22 mM glucose) or 6.3 (glucose). The results indicate that a large buffering capacity in the bath medium may be required to prevent excessive acidification of the restricted intercellular space. The apparent glucose induced alkalization of the intercellular space is difficult to

reconcile with the expected increased production of H during metabolism, but may be due to a change in the fixed charge of the islet matrix upon exposure to glucose or may reflect co-secretion of buffer molecules with insulin.

#### 6. Calcium Currents in Rat Pancreatic B-cells in Culture.

The patch voltage-clamp was used to measure  $\text{Ca}^{2+}$  currents across the membrane of rat pancreatic B-cells maintained in culture 2-10 days in CMRL-1066 (Gibco) with 5.6 mM glucose. Cultured cells secreted insulin in response to glucose (11-33 mM) and  $\text{K}^+$  (50 mM). Whole cell membrane currents (pipet solution (mM): 70 CsCl, 70 CsGlutamate, 10 CsPipes, 5 MgATP, 10 CsEGTA, pH=7) exhibited two components, an early transient one (Ca-1) and a delayed non-inactivating one (Ca-2). With bath solutions (mM: 120 CholineCl or TMA-Cl, 5 KCl, 1  $\text{MgCl}_2$ , 2-6  $\text{CaCl}_2$ , 5 CsHepes, pH=7.4), the mid-point for activation of Ca-1 was about -45 mV and for Ca-2 was 10 mV. With  $\text{Ba}^{2+}$  instead of  $\text{Ca}^{2+}$  in the bath, only Ca-2 was present. With  $\text{Ca}_0=4$  mM, the average maximum Ca-1 current was -75 pA and Ca-2 current was -125 pA ( $n=21$  I-V curves from 5 cells). Single  $\text{Ca}^{2+}$ -channel currents measured in the cell-attached configuration (pipet solution: 100 mM  $\text{Ca}^{2+}$  or  $\text{Ba}^{2+}$ ) with 11 mM glucose in the bath, ranged between 0.5 and 1.1 pA. Therefore, from the ratio "maximum whole-cell membrane current/single  $\text{Ca}^{2+}$ -channel current" (i.e. 200/0.5) we estimate that an islet B-cell is equipped with about 400  $\text{Ca}^{2+}$ -channels.

#### 7. Characterization of Potassium Channels in Pancreatic Beta Cells from ob/ob Mice.

Membrane potential of pancreatic islets from normal mice exhibits a characteristic oscillatory pattern, with bursts of action potentials correlated with extracellular glucose concentration. The calcium influx through voltage-activated calcium channels is thought to trigger the exocytosis of insulin containing granules. The control of membrane potential, by modulation of potassium channel activity, appears to be a critical step in the glucose-sensing mechanism in pancreatic beta cells. The ob/ob strain of mice displays a syndrome characterized by hyperglycemia and obesity. The electrical and secretory responses of the pancreatic islets to glucose show subtle differences with respect to normal mice. The patch clamp technique, in its cell-attached configuration was used. The bath solution contained (in mM) 135 NaCl, 4 KCl, 2  $\text{CaCl}_2$  and 10 Na-HEPES, pH 7.4. Pipet solution was 140 KCl and 10 K-HEPES, pH 7.4. At resting membrane potential three types of potassium channels were observed. The measured conductances were 20, 60 and 160 pS. The 60 pS channel activity was abolished by increasing extracellular glucose to 5 mM. This feature is similar to that of the normal B-cell ATP-sensitive potassium channel. The 160 pS channel shows a high activity, no voltage dependence, in contrast with the

characteristics of the 180 pS Ca-activated potassium channel of normal B-cells. This channel activity is also inhibited by 5 mM glucose. The differences could be involved in the different electrical behavior of ob/ob beta cells.

8. Glucose Sensitive Insulin Secretion Occurs Independent of Effects on Potassium Permeability and Membrane Potential in Mouse Islets of Langerhans.

The current view is that in pancreatic B-cells, glucose metabolism blocks K-channels. This depolarizes the membrane, activated voltage-gated  $\text{Ca}^{2+}$ -channels and triggers insulin release. AIM: To study glucose sensitive insulin secretion in the presence of a fixed depolarized membrane potential unresponsive to glucose (25 mM KCl), and compare this with conditions in which glucose depolarizes and produces the full range of electrical response (5 mM KCl). We studied acute-phase insulin release from microdissected, perfused mouse islets exposed to 10 minute increments of glucose from 5.6 to 33 mM and also during the first 10 minutes of a change from 5.6 mM to 27 mM. Secondary release was studied during 1 hour exposures to 27 mM glucose. 25 mM KCl significantly increased basal insulin secretion:  $220 \pm 21$  pg/min/6 islets ( $p < .05$ ). Acute-phase insulin release to stepwise increases of glucose was normal. Secondary phase insulin secretion was still glucose responsive. Fixing the membrane potential by blocking the K-ATP-channel with 4  $\mu\text{M}$  Glyburide also allowed for normal glucose sensitive insulin secretion. The data indicate that the effect of glucose to depolarize the membrane is not the mechanism which determines glucose-sensitive insulin secretion. The data are compatible with an effect of glucose to activate calcium channels via a voltage independent mechanism.

9.  $\text{Ba}^{2+}$ -induced Depolarization of Pancreatic B-cells is Correlated with Inhibition of Ca-activated But not ATP-Blockable, K-channels.

In mouse pancreatic B-cells, glucose reduces K-permeability, depolarizes the membrane, induces Ca-action potentials and increases insulin release.  $\text{Ba}^{2+}$  mimics these effects. In order to determine which K-channels are involved in glucose sensing, we studied the effects of  $\text{Ba}^{2+}$  on K-permeability in B-cells from mouse and rat using standard intracellular potential recording and patch clamp techniques.  $\text{Ba}^{2+}$  (0.5 - 5 mM) depolarized mouse B-cells, induced spikes and increased input resistance in the absence or presence of 2.5 mM  $\text{Ca}^{2+}$ . Spike duration increased with  $[\text{Ba}^{2+}]$ . In inside-out patches from cultured rat B-cells ( $\text{Na}^+$  in the pipette), 0.1 to 1 mM  $\text{Ba}^{2+}$  in the presence of 0.1 mM  $\text{Ca}^{2+}$  decreased the open probability of the Ca-activated K-channel,  $\text{K}(\text{Ca})$ , but did not inhibit the ATP-sensitive K-channel. The  $\text{K}(\text{Ca})$ -channel exhibited a sub-state conductance when  $\text{Ba}^{2+}$  and  $\text{Ca}^{2+}$  were present. In whole cell configuration,  $\text{Ba}^{2+}$  inward currents were larger than  $\text{Ca}^{2+}$

currents and did not inactivate.

10. Characterization and Control of Pulsatile Secretion of Insulin and Glucagon.

Periodic oscillation of insulin and glucagon by isolated mice islets has been studied. Pulsatile secretion of insulin and glucagon was observed at all glucose concentrations tested. The frequency of oscillation per 20 min for glucagon was  $5.0 \pm 0.26$  and for insulin  $4.0 \pm 0.26$  ( $n = 6$ ), approximating to periodicities of 4 and 5 min, respectively. These did not change by increasing the glucose concentration to 11.1 or 22.2 mM from 5.5 mM (basal). The maximal amplitude of glucagon secretion was not altered by raising the glucose concentration to 11.1 mM from basal. However, 22.2 mM glucose significantly suppressed the amount of glucagon released when compared with glucagon secretion in the presence of 5.5 mM glucose. In contrast, the maximal amplitude of insulin increased from  $444.2 \pm 37.7$  to  $777.2 \pm 61.4$  and from  $271.8 \pm 35$  to  $701 \pm 26.5$  pg/min ( $p < 0.01$ ,  $n = 6$ ) by switching from basal to 11.1 and 22.2 mM glucose, respectively. We conclude from this study that the pacemaker controlling pulsatile secretion of insulin and glucagon is within the islet. Although the amplitude of secretion of these hormones is regulated by the ambient glucose concentration, the frequency of their pulsatile secretion is not.

11. Composition of Secretory Granules by Electron Probe Microanalysis of Cryosectioned Rat Pancreatic Islets.

Electron probe microanalysis of islets allows variability as well as averages of granule composition to be assessed in both alpha and beta cells *in situ*. Islets were isolated by collagenase digestion from adult rats and incubated for 2 to 3 hours in culture medium at 37° C in the presence of 5.6 mmol/l glucose. Several islets were transferred to a Balzers freeze-fracture "hat" that was adapted with filter paper to absorb excess liquid. Within 20 seconds of transfer, the islets were frozen on the liquid helium-cooled block of a Medvac Cryopress freezing machine. Sections for analysis, 100 nm thick, were taken from within the first 20  $\mu$ m of the frozen surface. Analyses were obtained from approximately 50 nm diameter regions of the sample. Secretory granules in some cells contain high levels of sulphur and zinc, attributable to insulin-containing granules in beta cells. Secretory granules in other cells contain low levels of sulphur and no detectable zinc, attributable to glucagon-containing granules in alpha cells, which are found in high density at the surface of rat islets. The phosphorus content was high in granules of both alpha and beta cells, consistent with other observations that ATP is concentrated in secretory granules. Preliminary estimates indicate that the zinc content of beta granules is approximately 30 mmol/kg dry weight and the sulphur content approximately 300 mmol/kg dry weight, corresponding to approximately 50 mmol/kg dry weight insulin. No detectable zinc was found in the nucleus or cytoplasm. Calcium in granules from the same cell ranged from 10 to 50 mmol/kg dry weight, and in

nuclei was less than 2 mmol/kg dry weight. These data support the hypothesis that zinc and calcium are associated with insulin in granules of beta cells *in situ*.

#### IV. ADRENAL MEDULLARY ENDOTHELIAL CELLS.

##### 1. Steroid regulation of monoamine oxidase activity in the adrenal medulla.

Administration of different steroid hormones *in vivo* has distinct and specific effects on the MAO activity of the adrenal medulla. In an effort to reconstitute these effects in defined cells, we have isolated endothelial cells and chromaffin cells from the bovine adrenal medulla and tested each cell type for sensitivity to these steroids. As in the intact animal, we found that endothelial cell MAO activity was stimulated 1.5- to 2.5-fold by 10  $\mu$ M progesterone, hydrocortisone, and dexamethasone, inhibited by ca. 50% by 17- $\alpha$ -estradiol, but unaffected by testosterone. The type of MAO in the endothelial cells was found to be exclusively of the A type. The chromaffin cells had MAO B exclusively and were inert to treatment with dexamethasone. The mode of action of the various steroids on MAO A activity in endothelial cells seemed to be that of affecting the number of MAO molecules, as binding of [<sup>3</sup>H] pargyline, an MAO inhibitor, changed in proportion to changes in enzyme activity. Consistently, the kinetic parameters for MAO A showed changes in  $V_{max}$  but not  $K_m$  under all conditions. The specificity of steroid action on MAO A activity was also supported by the fact that steroid-induced changes in total cell division ([<sup>14</sup>C]thymidine incorporation) and total protein synthesis ([<sup>14</sup>C]leucine incorporation) were seen after changes in MAO A. We conclude that the differential effects of steroids on MAO activity in the intact adrenal medulla can be reproduced in cultured adrenal medullary endothelial cells but not in chromaffin cells. Therefore we suggest that the action of these steroid hormones on the intact adrenal medulla may be restricted to the endothelial cell component of this tissue.

##### 2. Interactions between PC12 cells and adrenal medullary endothelial cells in co-culture.

The adrenal medulla, in which clusters of chromaffin cells are enwrapped by microvasculature, is a classical model for studying basic issues of hormone and neurosecretion. To investigate the possible role of structural and functional interactions between chromaffin cells and capillary endothelial cells in regulating the development and secretory competence of the intact gland, we have established a model system, by co-culturing PC12 pheochromocytoma cells, a cell line derived from the rat adrenal medulla and bovine adrenal medullary endothelial (BAME) cells. The interactions between these two cell types are specific and primarily mediated via direct membrane-membrane contacts. Adhesion of PC12 cells to BAME cells is trypsin

sensitive, independent of extracellular calcium, but inhibited by serum proteins. In addition PC12 cells adhere preferentially to the surface of BAME cells and not to unrelated cells, like NIH 3T3 fibroblasts, or to the extracellular matrix derived from BAME cells. This is in contrast to preferential adherence to the extracellular matrix synthesized by non-related cells, such as corneal endothelial cells. Furthermore PC12-BAME adhesion can be inhibited by proteoliposomes prepared from cell surface extracts of both cells, but not from unrelated cells. These vesicular membrane extracts are enriched in several cell surface proteins, which might be relevant to the initial recognition/adherence events. Beyond the specific adhesion, co-cultures of PC12 and BAME cells, rapidly and mutually induce changes in cell-physiological and molecular-biological parameters. In both cells, expression of the protooncogene c-fos is enhanced within 30 minutes of contact indicative for alterations in their stages of differentiation. Following prolonged co-culture, the rate of proliferation in both cells is markedly decreased, while the rate of met-enkephalin immuno-reactivity in PC12 is significantly enhanced. In addition, the responsiveness of PC12 to NGF activation (viz. neurite outgrowth) is abolished. These effects cannot be inhibited by pretreatment of the cells with the relevant membrane extracts; however, they are absent when incubating PC12 cells with chemically fixed BAME cells. Preliminary experiments also indicate co-culturing with intact BAME cells, the activity of tyrosine hydroxylase and the secretory competence of PC12 cells is altered. While some of the mutual interactions can also be elicited by conditioned medium of the complementary cells, other effects, notably the changes in neuropeptide gene-expression, require direct cell-cell interactions between functionally intact cells. Our data suggest that following the rapid, specific adhesion between PC12 and BAME cells, both cells mutually modulate the physiology of the complementary cells either via direct transmembrane signalling and/or via synthesis and release of specific humoral factors. Thus our system might also serve as a suitable model for studying initial stages in organo-development and differentiation of secretory glands.

### 3. A Procedure for the Isolation and Culture of Adult Rat Brain Endothelial Cell-Containing Microvessel Fragments.

A number of laboratories have found the culture of endothelial cells from adult rat brain microvessels (MV) difficult to achieve. We therefore proceeded to develop a procedure for the isolation and culture of small endothelial cell-containing microvessel fragments (ECCMF). We followed traditional methods for isolation of MV. The vessels were purified through a dextran/percoll gradient and a nylon mesh was used to isolate the microvessel fraction. ECCMF were plated on fibronectin or gelatin coated dishes. Although growth of cells were observed, they did not stain for Factor VIII related antigen (FVIIIag). However, when the ECCMF were plated on Matrigel (basement membrane collagen), positive staining for FVIIIag, transferrin receptor and incorporation of low density lipoprotein was observed. Initial

studies were undertaken to test the viability of this preparation. Prostacyclin (PGI<sub>2</sub>) was measured after 24 hrs of equilibration as 6-Keto PGF<sub>1 $\alpha$</sub>  by a RIA. ECCMF maintained in media containing 20% FCS produced 5.89 ng/ml after 10 min. Incubation with bradykinin, thrombin and ionomycin induced a significant increase of PGI<sub>2</sub> production (104%, 223% and 88% respectively). It appears that this preparation will be useful for further studies on the effect of a variety of substances which are potentially active at the microvessel level, and will provide a basis for comparison with adrenal medullary endothelial cells.

## V. SECRETION FROM CELLS FROM THE CENTRAL NERVOUS SYSTEM.

### 1. The membrane of rat pineal cells in culture contains at least two types of potassium channels.

Potassium currents across the membrane of rat pineal cells in culture were measured using the patch clamp technique. Whole cell membrane currents (pipet solution (mM): 70 KCl, 70 Kaspargate, 10 K-Pipes, 5 Mg-ATP, 0.1 Na-EGTA at pH 7) exhibited both inward and outward currents. The analysis of the outward currents showed the presence of two kinetically different components, namely, an early transient and a delayed sustained current. In addition, the two components showed different steady-state characteristics and voltage sensitivity. The mid-point for activation of the early component (maximum chord conductance of 10.6 mS/cm<sup>2</sup>) was -5 mV, and that of the delayed component (maximum chord conductance 8.8 mS/cm<sup>2</sup>) was 20 mV. At the mid-point potential, while a 29 mV change in membrane potential induced an e-fold change in the magnitude of the transient component, a 41 mV change induced an e-fold change in the amplitude of the delayed component. The records of the single K-channel currents measured in the cell-attached mode (pipet solution (mM): 140 KCl, 1 MgCl<sub>2</sub>, 2.6 CaCl<sub>2</sub>, Na hepes at pH 7.4) also revealed the presence of two types of K-channels in the patches (K-1 and K-2). The single channel conductances and fractional open times at resting potential different (110 pS and 0.12 respectively for K-1; 148 pS and 0.95 for K-2). The K-1 channels alone were activated by exposure of the inside of the membrane to Ca<sup>2+</sup>; therefore we propose that the transient component of the whole cell membrane current is due to activation of the K-1 channels.

### 2. Two Types of Calcium Channels in the Plasma Membrane of the Pituitary Gonadotroph.

Measurements of the Ca<sup>2+</sup> currents in cultured rat pituitary gonadotrophs were made using patch clamp techniques. Analysis of the inward Ca<sup>2+</sup> currents, recorded in the presence of 5 mM Ca<sup>2+</sup> (or Ba<sup>2+</sup>), revealed a fast component, with activation-inactivation kinetics, and a delayed component with slower activation. The

rate of inactivation of the first component was found to be voltage dependent. At -44 mV, a 5.5 mV change in potential induced an e-fold change in the fraction of channels available to conduct  $\text{Ca}^{2+}$  current. During long-lasting (100-200 msec) low-frequency depolarizing voltage clamp pulses, the size of the delayed component of the  $\text{Ca}^{2+}$  current remained constant. A comparison of the time constants for turning off the  $\text{Ca}^{2+}$  conductance showed that with brief pulses the tail currents could be described as the sum of two exponentials. For pulses of long duration (100 msec) a single exponential fitted the time course of the current tails. The differential effects of membrane potential on inactivation, and the different time constants for turning off the  $\text{Ca}^{2+}$  conductance, suggest the presence of two types of calcium channels in the membrane of the gonadotroph. We propose that the  $\text{Ca}^{2+}$  channel with activation-inactivation properties plays a major role in the control of  $\text{Ca}^{2+}$  entry required for the physiological response of the cell.

### 3. Reconstitution of a calcium channel from of a highly purified preparation of mouse brain synaptosomes.

It is generally accepted that, regardless of the origin of the  $\text{Ca}^{2+}$  required for exocytotic release of neurotransmitters, intracellular  $\text{Ca}^{2+}$  stored play a fundamental role in secretion. Membrane channels provide the most efficient mechanism to furnish the  $\text{Ca}^{2+}$  required for release. However, the role of the endoplasmic reticulum (ER)  $\text{Ca}^{2+}$ -channels in nerve cells has not yet been elucidated. A highly purified synaptosome preparation was used to obtain intra-synaptosomal membranes. Application of the freeze and thaw method to the synaptosomes generated a membrane preparation rich in ER fragments.  $\text{Ca}^{2+}$ -channels were reconstituted into bilayers made with phosphatidylinositol (Avanti Polar Lipids). Membranes were formed at the tip of patch clamp pipets by the "double dip" method. A low  $[\text{Ca}^{2+}]$  solution (mM: 100 CsPipes, 1 CaHepes, pH = 6.4) was used in the chamber containing the membranes fragments (cis side) and a high  $[\text{Ca}^{2+}]$  solution in the pipet (mM: 100 CsPipes, 50 CaHepes, pH = 7.6). Under these conditions, we were able to incorporate  $\text{Ca}^{2+}$ -channels with the following properties: 1) Strong voltage sensitivity. Fractional open-time increased from 32.4 (measured at 20 mV) to 84.5% (at 60 mV). 2) Single  $\text{Ca}^{2+}$ -channel currents varied linearly with pipet potential to give a slope conductance of 20 pS. 3). The channel was activated by ATP (500  $\mu\text{M}$ ) and caffeine (10 mM) and was blocked by ruthenium red (10  $\mu\text{M}$ ). Nifedipine (10  $\mu\text{M}$ ) was without effect (drugs were added to the cis side). These pharmacological properties support the idea that the  $\text{Ca}^{2+}$ -channel originated from internal organelles such as the ER and emphasize its possible role in  $\text{Ca}^{2+}$  mobilization from internal stores.

4. A Possible Role for the Acetylcholine Transport System in Non-quantal Release of Acetylcholine at the Rodent Myoneural Junction.

The effects on the spontaneous, non-quantal release of acetylcholine (ACh) from motor nerve terminals of substances known to inhibit the ACh transport system present in cholinergic synaptic vesicles have been investigated. In mouse diaphragms, the hyperpolarization normally produced by d-tubocurarine (dTC) in muscle endplates that had been treated by an anticholinesterase was partly or completely abolished by 2-(4-phenylpiperidino) cyclohexanol (AH5183,  $10^{-7}$  -  $10^{-6}$ M), quinacrine ( $10^{-7}$ M) and tetraphenylborate ( $10^{-6}$ M). Since the sensitivity of the endplate to ACh was not changed, the block of the dTC induced hyperpolarization indicated an inhibition of the spontaneous, non-quantal release of ACh. This was confirmed by direct measurement of the ACh released by rat diaphragm. The release of ACh from the innervated diaphragm was decreased by about 50% by AH5183 ( $10^{-8}$  -  $10^{-6}$ M) and by 42% by quinacrine ( $10^{-7}$  -  $10^{-6}$ M). The ACh released was presumably neural, since the release of ACh from 4-day denervated diaphragms was not diminished by either AH5183 or quinacrine. The results indicate that the spontaneous release of ACh from the motor nerve terminals is probably mediated by a carrier which may be the vesicular transport system responsible for moving ACh into the vesicle. The transport system is likely to be incorporated into the membrane of the nerve terminal during exocytosis.

VI. MECHANISM OF REGULATION OF ION CHANNELS AND MUCIN SECRETION: THE PATHOPHYSIOLOGY OF CYSTIC FIBROSIS.

1. Regulation of Mucin Secretion From SW 1116 Human Colon Carcinoma Cells.

Secretion of mucin from SW 1116 human colon carcinoma cells were studied using the monoclonal antibody 19-9. This antibody has been previously shown to detect the mucin appearing in the plasma of cystic fibrosis patients. We have learned that SW 1116 cells secrete mucin constitutively and considerably in a temperature dependent manner. The basal secretion rate at 37°C is approximately 2-5  $\mu$ g/ $10^5$  cells/hr. By comparison, the mucin secretion rate from the frequently studied colon carcinoma cell line HT-29 is an order of magnitude lower than that of SW 1116 cells. Some common secretagogues, such as muscarine and forskolin, do not affect mucin secretion. Others, such as arachidonic acid, LTC<sub>4</sub> and pertussis toxin actually inhibit mucin secretion. However, the calcium ionophore A23187 induces a several fold increase in the mucin secretion rate. Finally, ultrastructural changes were observed in the golgi and in mucin secretion granules upon treatment of cells with LTC<sub>4</sub>. Chloride channels, studied by patch clamp methods, were identified in the plasma membrane of the cells. We anticipate that these studies

will provide insights into the relationship between mucin secretion and chloride channel activity, and contribute to our understanding of the cystic fibrosis phenotype in mucin secreting cells.

## 2. Regulation of Chloride and Potassium Channels in Mucin-Secreting SW 1116 Human Colon Carcinoma Cells.

SW 1116 cells secrete mucin and exhibit  $K^+$  and  $Cl^-$  channels of possible relevance to the regulation of the secretion process. The mucin is defined by the 19-9 antibody, previously shown to detect a mucin elevated in serum of patients with cystic fibrosis. Using the patch clamp technique (cell-attached), we observed two type of K-channels; pipet solution (mM): 140 KCl, 2.5  $CaCl_2$ , 1  $MgCl_2$ , 1.5  $MgCl_2$ , pH=7.4. The first type was seen occassionally and exhibits a large conductance ( $> 150$  pS), with fast activation and inactivation kinetics. In addition this K-channel exhibits anomalous rectifying properties. More frequently we observed a smaller conductance K-channel (20-24 pS), with sustained activity and voltage-independent fractional open time (0.15 from 20 to 70 mV pipet potential). The small channel was inhibited by muscarine (10  $\mu M$ ) and LTC<sub>4</sub> (10 ng/ml).  $Cl^-$ -channels were studied using the same solution in the pipet and the bath (mM): 140 NaCl, 2.5  $CaCl_2$ , 1.5  $MgCl_2$ , 10 NaHEPES, pH=7.4. Channel activity was rarely observed in the cell-attached configuration. In excised patches (inside-out), channel activity was frequently observed (symmetrical solutions), showing a non linear I-V relationship (at least two slope conductance, 120 pS and 30 pS), voltage dependence and insensitivity to 0.1, 1 and 10  $\mu M$  of NIPAB, a permeant, anthranilyc acid-related anion channel blocker. Positive identification as a  $Cl^-$ -channel was achieved by ion replacement procedures. Our intention is to relate ion channel regulation and mucin secretion.

## VII. PHOTSENSITIZED LABELLING: APPLICATION TO DRUG RESISTANCE IN CANCER CELLS AND GENERAL DETECTION OF INTRINSIC MEMBRANE PROTEINS.

### 1. Photosensitized Labelling: A Novel Methodology for Site Directed Labelling of Membrane Proteins.

[125-I]-5-Iodonaphthalene-1-azide (INA) is a lipophilic photolabile compound which proved efficient for selective labeling of lipid associated domains of membrane proteins. Recently, we introduced a new approach by which [125-I]-INA labeling of membrane proteins can be targeted to specific sites if photoactivation of the probe is induced by means of excitation energy transfer (photosensitization) from an energy donor chromophore. Photosensitized activation of [125-I]-INA is restricted to the vicinity of the donor chromophore because of the short effective range of the energy exchange process and hence, [125-I]-INA labeling will be confined to compartments defined by

the distribution of the chromophore. We demonstrate that using this technique we could obtain selective labeling of chromophore bearing proteins, and proteins nearest neighbor to chromophore tagged ligands in plasma membrane vesicles. Similarly, we show that this approach can identify in vivo membrane proteins which interact specifically with chemotherapeutic drugs in drug resistant tumor cells, and proteins which are located in the cell adjacent to fluorescently tagged phospholipid analogues. We suggest that this approach of photosensitized labeling (PSL) offers a wide range of possibilities for application in the study of the interaction between molecules in membrane and cells.

2. Detection of Nearest Neighbors to Specific Fluorescently Tagged Ligands in Rod Outer Segment and Lymphocyte Plasma Membranes by Photosensitization of 5-Iodonaphthyl 1-Azide.

Lima bean agglutinin-fluorescein 5-isothiocyanate conjugate (FluNCS-lima bean lectin) interacts with specific receptor molecules on membranes both from the rod outer segment (ROS) of the frog retina and from S49 mouse lymphoma cells. When [ $^{125}\text{I}$ ]-5-iodonaphthyl 1-azide ( $^{125}\text{I}$ -INA), which freely and randomly partitions into the lipid bilayer, is added to membranes and the suspension is irradiated at 480 nm, the FluNCS-conjugated lectin photosensitizes the [ $^{125}\text{I}$ ]INA but only at discrete sites. This results in the selective labeling of specific proteins: an 88-kDa protein on ROS membranes and a 56-kDa protein on S49 plasma membranes. Labeling is dependent upon the interaction of the FluNCS-lectin with glycosylated receptor sites, since N-acetylgalactosamine, but not methyl  $\alpha$ -mannoside, blocked labeling of the 56-kDa protein on S49 membranes. In contrast, a random labeling pattern of membrane proteins was observed upon irradiation at 480 nm using other fluorescein conjugates, such as FluNCS-bovine serum albumin (FluNCS-BSA) or FluNCS-soybean trypsin inhibitor (FluNCS-STI), which interact with cell membranes in a nonselective manner, or with N-(fluorescein-5-thiocarbamoyl)-n-undecylamine (FluNCS-NHC11), which is freely miscible in the membrane lipid. Random labeling was also obtained by direct photoexcitation of [ $^{125}\text{I}$ ]INA at 314 nm, with no distinct labeling of the 88- and 56-kDa proteins in the respective membranes. These results suggest that protein ligands can be used to guide sensitizers to discrete receptor sites and lead to their selective labeling by photosensitized activation of [ $^{125}\text{I}$ ]INA. Site-directed labeling is obtained by an amplification process that locally and time-dependently intensifies the radioactive signal, thus revealing minor membranal components that could not otherwise be visualized by random labeling. This approach provides a method that offers new possibilities for application in different fields of chemical and biological research.

### 3. Mechanism of Acquired Resistance to Methotrexate in Murine Leukemia Cells and in Their Doxorubicin-resistant subline.

The mechanisms of acquired resistance to MTX were studied in P388 murine leukemia cell lines that were sensitive or resistant to ADR. The rate of MTX accumulation in ADR-sensitive cells that have acquired resistance to MTX was found to be lower than that measured in cells that were sensitive to both drugs. Furthermore, in contrast to drug-sensitive cells, in the ADR-sensitive MTX-resistant cells, most of the intracellular MTX (86.2%) was bound and MTX polyglutamation was not detected. The initial rate of MTX accumulation in cells that were resistant to both drugs was comparable to that measured in cells that were sensitive to both drugs or that were resistant only to ADR. However, in the cells that were resistant to both drugs, the rate of MTX accumulation was maintained at its initial level for a period that was considerably longer than that found in the other cell lines. After 3 h of exposure to MTX, the accumulation of MTX in cells that were resistant to both drugs was fourfold higher than that measured in cells that were sensitive to both drugs. Furthermore, while 65 to 70% of the intracellular MTX was free, in cells sensitive to both drugs, or resistant only to ADR, the corresponding value in cells that were resistant to both drugs was <1.5%, and a much lower proportion of the MTX was polyglutamated. The sensitivity to TMQ of ADR-sensitive, MTX-resistant cells was similar to that found in cells that were sensitive to ADR and MTX. However, ADR-resistant cells, sensitive or resistant to MTX, were markedly resistant to TMQ. The sensitivity of ADR-resistant MTX-sensitive cells to TMQ was restored by the presence of 10  $\mu$ M verapamil. Such an effect was not observed in cells resistant to both drugs. It is suggested that P388 cells that have previously acquired resistance to ADR, when now selected by MTX, retain the MTX-transport system (in contrast to ADR-sensitive, MTX-resistant cells) and become resistant to MTX by increasing the activity of DHFR. The results obtained in ADR-resistant cells also suggested that resistance to TMQ was part of the multidrug resistance phenomenon.

## DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 21008-23 LCBG

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cytogenetics

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: J.H. Tjio

Chief, Section on Cytogenetics, LCBG, NIDDK

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1

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1

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Sex reversal studies with hormones and assessment of possible spontaneous sex reversal on several fish spp. Thus far no spontaneous sex reversal has been found in fish spp, which had been reported previously by several authors. The hormone treatment studies have only produced either sterile progenies or an increase in the progenies of females or males. Taxonomy studies of the family Anabadae are being continued to assess if there are karyotypic differences in species from different continents. 2. Elucidation of the kinetics of hemopoietic cells and the adverse effect of whole body irradiation on stem cell studies with Dr. G. Brecher. The use of CBA mice strains which carry different marker enzymes in all body cells of congenic sublines made it possible to determine the proportion of host and donor cells of both resting and dividing cells. This was determined by an efficient electrophoresis method. It is concluded that donor cells of larger inocula need undergo fewer divisions than cells of small inocula. It was observed that the number of LTRC rises more slowly in irradiated animals than CFU-S but preliminary data indicate as fast a turnover as in the CFU-S during the early post-irradiation period which indicate that L.T.R.C. are the most primitive cells. 3. Analysis of hyperdiploid CDS<sup>+</sup> (Lyl<sup>+</sup>) B cells in autoimmune NZB mice. Immunoregulation is being studied regarding why Lyl<sup>+</sup> B cells are critical in the development of autoimmunity, immuno-deficiency and B cell malignancy in cell transfer experiments. The hyperdiploid Lyl<sup>+</sup> B cells were transfused into irradiated autoimmune and non-autoimmune recipients. Chromosome markers show that only long lived donor cells found in the recipients donor cells are hyperdiploid Lyl<sup>+</sup> cells. The effect of *in vivo* transferred Lyl<sup>+</sup> B cells on recipients' antibody production was observed. It was found that Lyl<sup>+</sup> B cells are immunoregulatory and recipients with the *x.i.d.* gene can be used to assess the role of hyperdiploid Lyl<sup>+</sup> B cells on autoantibody formation. 4. Post and prenatal diagnosis of karyotypic aberration studies and the genomic diagnosis of hemophilia A by R.F.L.P. analysis are being continued.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 21019-07 LCBG

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mechanism of hormone and transmitter secretion

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Harvey B. Pollard, Chief, Laboratory of Cell Biology and Genetics, NIDDK.  
 Others: G. Lee, Ph.D., Res. Chemist; E. Rojas, Ph.D., VS; I. Atwater, Ph.D., Expert;  
 M. Levine, M.D., Sen. Inv., Med. Off.; E. Forsberg, Ph.D., SF; A. Burns, Ph.D., Expert;  
 M. Srivastava, Ph.D., GW; C. McCutchen, Ph.D., Res. Phys.; G. Kuipers, Ph.D., VF;  
 K. Magendzo-Weinberger, Ph.D., SV; A. Munoz, M.D., SV; V. Cena, Ph.D., M.D.; M. Li,  
 M.D., VF; M. Shirvan, Ph.D., IRTA; A. Shirvan, Ph.D., IRTA; Y. Raviv, Ph.D., VF; M.  
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 M.D., VF; A. Goncalves, Ph.D., VA; A. Moura, Ph.D., VF; G. Goping, EM Tech.; P.  
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- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Our recent work has focussed on the processes leading to fusion between granule and plasma membranes during exocytosis in cells such as chromaffin cells, beta cells from islets of Langerhans, nerve terminals, and mucin secreting human colon carcino-ma cells. Electron microprobe analysis of fast frozen chromaffin cells has shown that isolated granules have lost substantial amount of potassium, but gained sodium. Both barium and calcium induce secretion from chromaffin cells, but we now learn they do so by separate and additive mechanisms. Protein kinase C, which may regulate secretion, binds not only to plasma membranes but also to secretory granule membranes in a calcium dependent manner. The secretory event may depend on the pH within the secretory granule. Calcium enters chromaffin cells through several voltage sensitive pathways, one of which is insensitive to both dihydropyridines and w-conotoxin. Using whole cell recording techniques we calculate that secretion of one granule requires 120,000 calcium atoms. We know it to form highly selective, voltage gated calcium channels in phosphatidylserine bilayers or biological membranes. We have cloned the synexin cDNA, and have learned that it is homologous with the class of calcium dependent membrane binding proteins (AKA, annexins, lipocortins, endonexin fold proteins). The mechanism of membrane fusion may involve formation of a hydrophobic bridge of synexin molecules between fusion partners. Secretion of insulin from human B-cells involves three K channels, similar to those found in rat B-cells. The pH of the cytosol, modified by glucose and bicarbonate, regulates insulin secretion from B-cells. Endothelial cells were found to express MAO-A in a steroid hormone sensitive manner, and to interact with PC12 cells. Endothelial cells from rat brain microvessels were isolated and found to synthesize and secrete prostacyclin in response to bradykinin and other agonists. Rat pineal cells in culture were found to express at least two K channels, while two different calcium channels were detected in the plasma membrane of pituitary gonadotrophs. We also reconstituted a calcium channel from brain ER derived from highly purified synaptosomes. Mucin secretion was measured from human colon carcinoma Sw610 cells, and chloride channel activity measured in parallel cultures. Photosensitized labeling has been used to study multidrug resistance in cancer cells, and to label specific membrane proteins.

ANNUAL REPORT OF THE LABORATORY OF BIOCHEMICAL PHARMACOLOGY  
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY  
DISEASES

I. POLYAMINES

Polyamines are major cellular components and have been shown to be involved in many systems related to growth and differentiation. Our studies have been directed towards elucidating the physiological function, biosynthesis, and regulation of these amines. Most of our current studies have been directed toward a study of *S*-adenosylmethionine decarboxylase, which is a key enzyme in the biosynthesis of spermidine and of spermine in prokaryote and eukaryote cells. This enzyme has a most unusual structure, as it contains a covalently linked pyruvoyl moiety which is required for enzyme activity. This enzyme is coded for by the *speD* gene in *Escherichia coli* and *SPE2* in *Saccharomyces cerevisiae*.

*Escherichia coli*. We have extended our studies on the nucleotide sequence of the *speED* operon and have shown that the *speED* operon is much longer than anticipated. Upstream from the coding region for the *speE* gene product is a 574 bp nucleotide moiety which contains a promoter and an additional open reading frame. The latter codes for a 12,000  $M_r$  protein of unknown function.

. . . . . Drs. Q.-W. Xie, C. W. Tabor, and H. Tabor

We have previously shown that the enzyme *S*-adenosylmethionine decarboxylase is first formed as a proenzyme that is processed by a post-translational cleavage reaction at the Lys111Ser112 peptide bond. We have now developed a cell-free system for studying this processing, and have shown by site-specific mutagenesis that changes in the Lys111 amino acid markedly decrease the cleavage reaction. The cleavage reaction requires sulfhydryl groups and is completely inhibited by tetrathionate; the inhibition is reversed by dithiothreitol. This reversible inhibition and reactivation has permitted the isolation and purification of the proenzyme and a study of the characteristics of the cleavage reaction.

. . . . . Drs. C. W. Tabor and H. Tabor

We have also continued our studies on the phenotypic characteristics of our amine-deficient mutants. With Dr. K. W. Minton (Uniformed Services University of the Health Sciences), we have shown that the amine-deficient mutants are strikingly more sensitive to the toxic effects of paraquat than the parent strains. Paraquat is a known source of superoxide, and it is possible that these new findings are related to our previous findings that glutathionylspermidine is present in *E. coli*, and this glutathione derivative would be absent in amine-deficient cells.

. . . . . Drs. K. W. Minton, C. W. Tabor, and H. Tabor

*Saccharomyces cerevisiae*. We have previously shown that *S*-adenosylmethionine decarboxylase from *S. cerevisiae* also has a pyruvoyl end group that is essential for activity, even though the enzyme from this source appeared to have different characteristics from the *E. coli* enzyme. Therefore, it was of interest to study the sequence of the gene coding for this enzyme in *S. cerevisiae*, as well as the structure and biosynthesis of the protein.

The *SPE2* gene was cloned into overproducing plasmids and expressed in both yeast and *E. coli*. Pulse-chase experiments showed that the yeast enzyme is first synthesized as a proenzyme and then is cleaved post-translationally into subunits of molecular weight 10,000 and 36,000. The amino acid sequence of these subunits, when compared with the amino acid sequence deduced from the nucleotide sequence, showed that the cleavage site is at the Glu87Ser88 peptide. The primary structures of the yeast and *E. coli* enzymes are completely different.

. . . . . Drs. K. Kashiwagi, C. W. Tabor, and H. Tabor

The chromosomal localization of *SPE1* (ornithine decarboxylase) is on chromosome XI, very close to the *LAP1* gene.

. . . . . Drs. Q.-W. Xie, C. W. Tabor, and H. Tabor

## II. YEAST RNA VIROLOGY

There are five families of double-stranded RNA (dsRNA) that replicate in cells of the yeast *Saccharomyces cerevisiae*. Three of these, L-A, L-BC, and M, replicate in viral particles. A single-stranded RNA, called 20S RNA, also replicates in yeast.

We have developed an *in vitro* L-A RNA replication system (Drs Fujimura and Wickner) and determined the essential sites on L-A (+) strands for this reaction (Drs Esteban, Fujimura, and Wickner). They are the 3' end 4 bases, a stem-loop structure adjacent to these 4 bases, and an internal site (the Internal Replication Enhancer or IRE). Overlapping with the IRE is a site needed for binding to viral particles of (+) single strands (Viral Binding Site or VBS). The VBS includes a stem-loop structure with an A residue protruding on the 5' side.

. . . . . Drs. R. Esteban, T. Fujimura, and R. B. Wickner

We have developed an *in vitro* transcription system ((+) strand synthesis from dsRNA template) which we can use to study the template requirements of this reaction as well (Drs. Fujimura and Wickner). This system consists of opened empty viral particles and carries out the conservative, template-specific synthesis of viral (+) strands. This is the only dsRNA virus for which template-dependent *in vitro* systems have been developed. L-A encodes a fusion protein whose N-terminus is a major coat protein monomer and whose C-terminus has homology with the RNA-dependent RNA polymerases of (+) strand RNA viruses. We have evidence that this protein is formed by ribosomal frameshifting (Drs. Dinman and Wickner).

. . . . . Drs. T. Fujimura, J. D. Dinman, and R. B. Wickner

We have cloned the chromosomal *MAK10* gene (Drs. Y.-J. Lee and Wickner), needed by the L-A virus for its replication, and are studying its structure and regulation. We have isolated a clone of a chromosomal gene that when present on a high copy vector makes *ski*<sup>-</sup> strains no longer grow slowly, that is, these strains are no longer sensitive to the viral pathogenicity otherwise associated with derepression of viral

replication that results from a *ski*<sup>-</sup> mutation (Drs. H.-S. Lee and Wickner).

. . . . . Drs. H.-S. Lee, Y.-J. Lee, J. C. Tercero-Lopez, and R. B. Wickner

We have isolated clones of most of the 20S ssRNA replicon and obtained evidence that it is a circular RNA replicon, like the human hepatitis delta virus.

. . . . . Drs. Y. Matsumoto and R. B. Wickner

### III. NUCLEIC ACIDS

#### L1 Elements

*Introduction.* L1 DNA (long interspersed repeated DNA, LINE 1 DNA) is a ubiquitous feature of mammals and comprises at least 10-20% of their genomes. L1 elements contain a promoter-like sequence at the left end, two highly conserved open reading frames (ORFs), and a guanine-rich polypurine/polypyrimidine sequence near the right terminus. Amplification of L1 elements has occurred repeatedly during mammalian evolution, and invasion by L1 elements into new sites is a frequent cause of polymorphism in mammals including humans. This can occur during the lifetime of the individual as was dramatically illustrated by a case of factor VIII deficiency due to L1 insertion in the factor VIII gene of a child whose parents both had the normal gene. We have been studying the L1 family of rats and describe below our recent findings.

*Current Findings.* We previously showed that the guanine-rich polypurine:polypyrimidine tract at the right end of an L1 element destabilizes contiguous duplex DNA sufficiently so that it hybridizes homologous single-stranded DNA which can then act as a primers for DNA synthesis. We now find that this tract adopts several non-B DNA structures *in vitro* that may explain this phenomenon. At pH 5.0 in the presence of Mg<sup>2+</sup>, two types of triplexes are formed: a G·G·C triplex, and an unusual C·G·C triplex which contains a hairpin structure in the free purine strand. At pH 7.5 little or no triplex is observed although this region is still quite reactive with probes of non-B DNA structure. Therefore the duplex in the region of the L1 polypurine:polypyrimidine tract is distorted which we speculate can account for the above results and also contribute to the recombinogenicity observed for this region *in vivo*.

Three randomly chosen L1 insertion sites adopt abnormal DNA structures as well which might facilitate targeting of L1 elements to these regions. When the right end of the L1Rn3 element and its target site are present within the same topological domain, they compete for available supercoil energy. The amount of non-B DNA formed at each site varies with pH, the concentration of cations, and the size of the topological domain. *In vivo* competition between the two sites for supercoil energy might modulate the supercoil-dependent properties of L1 elements and their target sites.

We are now examining the effect of both the L1 and target site non-B DNA forming regions on illegitimate recombination *in vivo*. To do this plasmids containing one or both of these sites are propagated in a suit-

able mammalian cell and their progeny are examined for recombinationsl events.

. . . . . Drs. K. Usdin and A. V. Furano

One of the most intriguing features of L1 families is their repeated acquisition of novel promoter-like sequences during evolution. Our previous finding that the rat L1 promoter-like region is in fact a very active promoter was the first evidence that L1 DNA is not just some non-functional relic DNA. We have further characterized this promoter by transcriptional mapping, deletional analysis, its interaction with other regulatory DNA sequences, and nuclear protein factors.

The major transcripts most likely begin about 300 bp from the 5' end of the sequence which indicates that a full length transcript of the L1 element cannot be made from a linear template unless it contains active tandem promoters. Deletion analysis showed that the 650 bp promoter consists of both inhibitory and stimulatory modules. For example, deletion of the first 150 bp completely abolishes promoter activity, but deletion of the next 200 bp restores significant activity. The interaction of these modules with other regulatory sequences and protein transcriptional factors is now under way (also see below).

. . . . . Drs. E. Pascale and A. V. Furano

The intact L1 promoter exhibits a very strong (> 20-fold) synergy with the SV40 promoter sequence. This was found in several cell lines, and *in situ* assays using antibodies to the gene product produced in these experiments showed that the number of cells capable of forming active transcriptional complexes was very much greater when both the L1 and SV40 promoters were present than when either were introduced into cells alone. Since the SV40 promoter alone is one of the strongest promoters described, these results are quite provocative. Subclones of the SV40 promoter region are now being tested with the full length L1 promoter and its various modules in order to determine precisely what sequences are responsible for this interaction.

. . . . . Drs. E. Valle, E. Pascale, and A. V. Furano

Gel retardation experiments with crude nuclear extracts show that numerous specific DNA:protein complexes are formed between both the inhibitory and stimulatory promoter modules described above. We are now determining at the nucleotide level which sequences are involved in order to purify and characterize these transcriptional factors.

. . . . . Drs. B. E. Hayward and A. V. Furano

*L1 Encoded Proteins.* The sequences of L1 ORFs are very highly conserved, suggesting that they have been under selective pressure for L1 function. Using genomic DNA enriched for L1 sequences we are now cloning these L1 protein encoding sequences as fusion proteins with *E.coli*  $\beta$  galactosidase. Determining the function of the L1 proteins is essential for understanding the biological properties of these elements.

. . . . . Drs. B. E. Hayward and A. V. Furano

*Ancestral L1 Elements.* Although the present day mammalian families are each the product of an independent amplification event that was appar-

ently limited to individual species, this does not prove that repeated rounds of amplification has occurred in the past. However, we have recently isolated two L elements, which we call Lx and Ly, that clearly bear an ancestral relationship to the present day L1 families in mouse and rat, are as prevalent in mouse as they are in rat, and, in the case of Lx, is almost as prevalent in the rat genome as is the present day L1. We have no evidence yet that intact members of Lx or Ly are present in rats and we presume that these sequences are relics of earlier highly amplified ancestral L families that have been somehow replaced by their present day counterparts. Therefore it seems quite likely that L DNA has been repeatedly amplified in the past and there is no reason to suppose that it can happen again with presumably dire consequences for the species. We are now determining the genealogy of these ancestral L elements in mouse and rat species to determine how long ago Lx and Ly were amplified.

. . . . . Drs. E. Pascale, E. Valle, and, A. V. Furano

We are studying the *Escherichia coli* bacteriophage T4 as a model system for duplex DNA replication. Efficient DNA replication *in vitro* is achieved with seven purified proteins encoded by T4 phage: T4 DNA polymerase (gene 43 product), gene 32 DNA helix-destabilizing protein, the gene 44/62 and gene 45 polymerase accessory proteins, and the genes 41 and 61 proteins which function together as a primase and as a DNA unwinding enzyme (or helicase).

*Interactions between the T4 Replication Proteins at the Replication Fork.* Since the same seven T4 replication proteins are responsible for synthesis on the leading and lagging strands, it is essential that they be able to distinguish between a forked DNA template where continuous strand displacement synthesis is required, and the lagging strand template where it is important not to displace the preceding Okazaki fragment. Using synthetic model templates we have shown that completely complementary DNA analogous to the preceding discontinuous fragment is not displaced, but that a fork of 50 nucleotides is sufficient to allow rapid strand displacement synthesis on the leading strand. Either the 32 ssDNA binding protein or the 41 protein helicase is required for strand displacement synthesis. *In vitro* there is a significant lag time in establishing the 41 protein-dependent rapid synthesis which is not decreased by increasing the length of the fork on the side of the lagging strand template up to 555 nucleotides.

. . . . . R. L. Ellis and Dr. N. G. Nossal

Our recent studies with forked DNA templates indicate that the average size of Okazaki fragments made *in vitro* increases (and the number of new starts decreases) when either the 61 protein primase component or the DNA polymerase is limiting during the initial stage of lagging strand synthesis. The average length of 1.5 kb *in vivo* and under nonlimiting conditions *in vitro* increased to more than 5 kb at lower primase and polymerase concentrations which did not decrease the rate of continuous synthesis on the leading strand. While fragment size is dependent on the initial concentration of polymerase and primase, once 41 protein-dependent leading and lagging strand synthesis is established, 10-fold dilution into reaction mixtures lacking only 61 protein or polymerase gives fragment sizes close to those made with the initial high concentrations of these enzymes. Our tentative interpretation of these results is that leading and lagging strand synthesis are uncoupled dur-

ing the initial stage of synthesis *in vitro* so that fragment initiation depends on the simultaneous binding of 61 protein, polymerase, and the accessory proteins. However, the dilution experiments suggest that once coupled synthesis is established, the polymerase and 61 protein remain bound to the lagging strand template to a significant extent during successive rounds of synthesis of Okazaki fragments.

. . . . . Dr. N. G. Nossal

*Ribonuclease H Activity in T4 Infected Cells Removes the RNA Primers on the Lagging Strand.* We have previously shown that an RNase H activity increases after T4 phage infection, and have now extensively purified this protein from T4 infected *E. coli* cells. The purified enzyme can completely remove the pentamer RNA primers (pppApCpNpNpN) from DNA made by the T4 replication proteins *in vitro*. Addition of T4 RNase H and T4 DNA ligase to the seven other replication proteins leads to extensive joining of adjacent discontinuous fragments, indicating that these enzymes are sufficient for primer release, gap filling, and DNA chain sealing on the lagging strand.

The RNase H activity increases early after infection simultaneously with many phage-encoded replication enzymes, but it is not clear if the phage encodes a new RNase H or activates a host enzyme. The N-terminus of a 33 kd protein copurifying with the RNase H activity matches that encoded by a predicted open reading frame of the same size on the T4 genome. We are currently cloning the T4 DNA containing this open reading frame to determine whether it encodes the RNase H protein.

. . . . . Drs. H. C. Hollingsworth and N. G. Nossal

*Essential Regions of T4 DNA Polymerase.* T4 DNA polymerase has six regions which share sequence similarity with corresponding regions in a family of DNA polymerases including eukaryotic cellular DNA polymerase  $\alpha$  and the Herpes, Epstein-Barr, and Vaccinia Virus DNA polymerases. These similar regions are separated by regions varying in length and amino acid sequences in the different polymerases. To determine the function of the similar and intervening regions in this family of polymerases, we have begun to characterize altered T4 DNA polymerases produced by site-specific mutagenesis of the cloned gene.

. . . . . Drs. P. Spacciapoli and N. G. Nossal

*Bacteriophage T4 Gene Expression.* Throughout infection, bacteriophage T4 programs the temporal expression of early, middle, and late genes. As a model for examining how T4 regulates middle RNA, we are focusing on the expression of a cluster of T4 prereplicative genes located 5'  $\rightarrow$  3': *X.1* (newly identified gene of unknown function, see below), *uvsX* (recombination protein), 40 (stimulates head formation), and 41 (primase-helicase component).

Our mapping of *X.1-uvsX-40-41* transcripts shows that the expression of this region arises from polycistronic, middle messages whose major 5' and 3' ends are consistent with phage regulation at the level of transcription initiation and elongation or processing. Major 5' ends, 200 and 900 bases upstream of *uvsX*, show homology with transcription initiation sites dependent on the T4 transcription factor *motA* and are not observed to any significant degree after transcription with the host RNA polymerase lacking phage modifications. The major 3' end of *uvsX* RNA

maps just after the end of *uvvX*, immediately downstream of a GC-rich hairpin. This end apparently arises from factor-dependent transcription termination or processing since it is observed in RNA expressed by a *X.1-uvvX-40-41* plasmid *in vivo*, but it is not seen after *in vitro* transcription with purified RNA polymerase.

The *E. coli* transcription termination (*rho*) mutant *rho026* is a *rho* mutation whose terminating activity is not effectively overcome by phage lambda antitermination. During an abortive T4 infection of *rho026*, the level of 41 protein is depressed; a T4 mutant in *goF* gives a wild type amount of 41. Our RNA analyses demonstrate that the several-fold decrease in 41 protein is accompanied by a similar decrease in 41 RNA. This is due in part to more of the upstream *uvvX* RNA ending at the mapped 3' end. In an T4 *goF1/rho026* infection, the relative amount of RNA reading into 41 versus that stopped is close to wild type. These results are consistent with the presence of a termination or processing site downstream of *uvvX* whose use involves host *rho* and T4 *goF*.

The 840 bp of DNA immediately upstream of *uvvX* is genetically unmapped, but is co-expressed with *uvvX*, 40 and 41. Our DNA sequence analysis reveals a 221 amino acid open reading frame, designated *X.1*, which shares a region of similarity with 2 other T4 genes: gene 69 (putative replication protein) and the ORF 5 (unknown function) upstream of *tRNA Arg*. *In vitro* transcription/translation of plasmids and restriction fragments including the *X.1* region yields a protein of ~25,000 daltons, consistent with expression of the *X.1* protein.

. . . . . R. L. Ellis and Dr. D. M. Hinton

*Hepatitis Non-A, Non-B*. Hepatitis non-A, non-B (HNANB) is a world-wide problem, and 90% of the transfusion-related hepatitis cases in the United States (and 80-90% in several other countries) are diagnosed as HNANB. Approximately 50% of all acute HNANB patients develop chronic HNANB (an estimate of 4 million persons). They remain as potential sources of infection. Recent publications suggest a correlation between certain hepatocellular carcinomas and chronic HNANB infections.

Based on biochemical, immunological, and morphological evidence, we suggested that the HNANB agent is a mammalian type C retrovirus or retrovirus-like (i.e., a positive stranded RNA virus). Recently, using an *in vitro* focus-induction assay developed for mammalian type C viruses, we observed that pelleted material from HNANB sera (transfusion-related) induced foci formation. This result is consistent with the presence of a mammalian type C virus in HNANB sera.

A DNA probe of 757 base pairs isolated from HNANB-infected chimpanzee liver and selected by subtractive hybridization with normal chimpanzee liver was shown to hybridize *in situ* with liver sections from three HNANB-infected chimpanzees but not with liver from two HBV-infected animals. This DNA fragment has been cloned, completely sequenced, and placed under the control of the Sp6 and T7 promoters. Sequence data indicated that the DNA fragment is 757 base pairs. The vector pBR322 and the 757 base fragment has been designated pSC22.

Cultures of peripheral blood mononuclear cells isolated from a HNANB patient (R.F.) was found to hybridize to the 757 base pair probe. Also, cultures of the mink lung cell line (CCL64.1) superinfected with HNANB

viral particles (from 15 patients) hybridize specifically with the 757 base probe. These experiments are still in progress.

. . . . . Drs. W. G. Coleman, Jr., A. W. Gordon, and L. Chen; B. P. Seto, (HL, DBBP, Center for Biologics Evaluation and Research)

#### IV. MEMBRANE STUDIES OF MACROPHAGES AND OF *ESCHERICHIA COLI*

**Aldoheptose Biosynthesis.** Previously, a novobiocin-hypersensitive mutant of *Escherichia coli* K-12 carrying a *cysE-pyrE* linked mutation, designated *rfaD*, which specifically affects the synthesis of the aldoheptose, L-glycero-D-mannoheptose, has been isolated and genetically characterized. The *rfaD* gene codes for ADP-L-glycero-D-mannoheptose-6-epimerase, an enzyme required for lipopolysaccharide (LPS) core biosynthesis. The nucleotide ADP-D-glycero-D-mannoheptose accumulates in *rfaD* mutant strains. The *rfaD* phenotype includes increased permeability to a large number of hydrophobic drugs and dyes, and the formation of mucoid colonies. A 9-kilobase DNA *EcoRI* fragment carrying the *rfaD* gene was initially identified in the Clarke-Carbon Colony Bank cloned in pBR322, and subsequently smaller restriction fragments were cloned into several expression plasmid vectors. The proteins expressed by *RfaD*<sup>+</sup> plasmids, using several *in vivo* and *in vitro* expression systems, have been examined by SDS gel electrophoresis. *RfaD*<sup>+</sup> plasmids express a protein with a molecular weight of 37,000. Finally, ADP-L-glycero-D-mannoheptose-6-epimerase has been purified to homogeneity and partially characterized. The entire *rfaD* gene is located on a 1.3 kilobase *SspI-HprI* fragment. This region and flanking regions have been completely sequenced, and the coding region has been determined.

. . . . . Drs. J. C. Pegues, L. Chen, A. W. Gordon, and W. G. Coleman, Jr.

#### V. ENZYME MECHANISM AND PROTEIN STRUCTURE

**Three-Dimensional Structure of the Tryptophan Synthase  $\alpha_2\beta_2$  Multienzyme Complex from *Salmonella typhimurium*.** We have determined the structure of the tryptophan synthase multienzyme complex by x-ray crystallography at 2.5 Å resolution. The structure shows the folding pattern of the  $\alpha$  and  $\beta$  subunits, the arrangement of the subunits in the complex, the residues in each of the active sites, and the presence of a tunnel 25 Å long that connects the active sites. Analyses of the crystal structure and computer modeling clarify the geometry of residues 175 and 211 in the substrate binding site of the wild type  $\alpha$  subunit and in mutant forms with single or double alterations at these positions. X-Ray crystallography of mutant and wild type forms of the enzyme in the presence and absence of ligands is in progress.

. . . . . Drs. E. W. Miles and S. A. Ahmed with Drs. C. C. Hyde, E. A. Padlan, and D. R. Davies (LMB, NIDDK)

**Microspectrophotometric Studies on Single Crystals of the Tryptophan Synthase  $\alpha_2\beta_2$  Multienzyme Complex from *Salmonella typhimurium*.** Our studies demonstrate that chromophoric intermediates are formed between pyridoxal phosphate and substrates at the active site of the  $\beta$  subunit in the crystal and that ligand-dependent site-site interactions occur in the crystal. These studies demonstrate that the enzyme is active in the

crystalline state and provide information for x-ray crystallographic studies in the presence of ligands.

. . . . . Drs. E. W. Miles and S. A. Ahmed with Drs. A. Mozzarelli, A. Peracchi, and G. L. Rossi (University of Parma, Italy)

*Detection and Identification of Transient Intermediates in Reactions of Tryptophan Synthase.* We are using rapid-scanning stopped-flow spectroscopy to determine the rates of formation and decay of transient intermediates in the reactions of tryptophan synthase. Studies with two reaction intermediate analogs, oxindolyl-L-alanine and 2,3-dihydro-L-tryptophan, have clarified the mechanism of reaction and demonstrated the occurrence of a previously undetected tetrahedral (*gem*-diamine) intermediate. We are currently investigating the mechanism of channeling of indole in studies using wild type and mutant forms of tryptophan synthase.

. . . . . Drs. E. W. Miles and Y. Sawa with Dr. M. F. Dunn (University of California, Riverside)

*Site-Directed Mutagenesis of the Tryptophan Synthase  $\alpha_2\beta_2$  Complex.* We are using recombinant DNA techniques to modify the *trpA* and *trpB* genes from *S. typhimurium*. Wild type and mutant forms of the  $\alpha_2\beta_2$  complex from *S. typhimurium* are expressed in very high yield and are crystallized from crude extracts by addition of poly(ethylene glycol) and spermine. Studies of mutant forms of the  $\alpha$  subunit with a series of single amino acid replacements of glutamic acid 49 and aspartic acid 60 provide strong evidence that these two residues are catalytic bases. A role for each residue is proposed on the basis of these results and on the location of the residue in the crystal structure. Parallel studies demonstrate that another active site residue, tyrosine 175, is not essential for catalysis. The results of mutation of the  $\beta$  subunit at residues histidine 86, arginine 148, and cysteine 230 show that these three residues are not essential for catalysis or substrate binding. Studies of amino acid substitution of lysine 87, which forms a Schiff base with pyridoxal phosphate in the wild type  $\beta$  subunit, provide evidence that the catalytic base in the  $\beta$  subunit which removes the  $\alpha$  proton of L-serine is lysine 87, not histidine 86, as previously proposed. We are currently investigating the catalytic roles of glutamic acid residues 109 and 350, residues which are located in the pyridoxal binding site of the  $\beta$  subunit. We are also determining the effect of single amino acid replacements at position 60 of the  $\alpha$  subunit on the conformational stability of this subunit. Crystals of some of the mutant forms have been prepared for analysis by x-ray crystallography.

. . . . . Drs. E. W. Miles, H. Kawasaki, S. A. Ahmed, H. Morita, H. Morita, S. Nagata, H. Kanzaki, Y. Sawa, and A. Kayastha with Dr. Peter McPhie (LBM, NIDDK) and Dr. K. Yutani (Institute for Protein Research, Osaka University, Japan).

The interaction between fibrous rabbit muscle actin and the globular proteins aldolase and bovine serum albumin has been studied by sedimentation equilibrium and electron microscopy.

. . . . . S. Lakatos and A. P. Minton

The PC-based data acquisition and control system for the analytical ultracentrifuge, which was initially developed and installed last year,

has been substantially upgraded with new hardware and extensively revised software.

. . . . . Drs. A. P. Minton and M. S. Lewis (BEIB, DRS)

The ultracentrifuge simulation program has been extended to treat the case of concentrated macrosolutes, the sedimentation of which cannot be validly described in the ideal approximation.

. . . . . Drs. R. C. Chatelier (formerly LBP, NIDDK; now Commonwealth Scientific and Industrial Research Organization, Melbourne, Australia) and A. P. Minton

Models for estimation of the fraction of cytoplasmic volume that may be considered adjacent either to boundary membranes or to the surface of fibrous elements comprising the cytomatrix have been formulated.

. . . . . Dr. A. P. Minton

During the past year the reductive activation of the valyl-tRNA synthetase studied was found to be reversible. The enzyme has been observed to oscillate between an active and an inactive form, and the oscillation is affected by several ligands. The implication of this finding is that the reductive process, which regulates the synthetase, is itself regulated, and a cascade of reactions must thus operate to control the rate of the first step of protein biosynthesis.

. . . . . Dr. S. Black

Cooperative binding systems are being studied taking into account site or subunit interactions, ligand interactions, aggregation and redistribution in proteins, and model systems. Methods are being developed to evaluate reasonable values for the parameters describing these systems. Specific studies are being done on the fibrinogen-thrombin clotting mechanism.

Amino acid sequences of proteins are analyzed primarily with the Monte Carlo techniques to evaluate the uniqueness and homology of these sequences. The property of uniqueness (the occurrence of a *small* peptide at a frequency considerably less than that expected) has been quantified, and speculation on this quantity and the immune response are under investigation.

. . . . . Drs. H. A. Saroff and E. Mihalyi

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 23,140-31 LBP
PERIOD COVERED October 1, 1988      to      September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Biochemistry of Sulfur-Containing Compounds		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)  <div style="display: flex; justify-content: space-between;"> <span>PI:      Simon Black, Ph.D.</span> <span>Chemist (Research)</span> <span>LBP NIDDK</span> </div>		
COOPERATING UNITS (if any)  None		
LAB/BRANCH Laboratory of Biochemical Pharmacology		
SECTION Section on Pharmacology		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:      1.3	PROFESSIONAL:      1.0	OTHER:      0.3
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div> <input type="checkbox"/> (a) Human subjects  <input type="checkbox"/> (a1) Minors  <input type="checkbox"/> (a2) Interviews         </div> <div> <input type="checkbox"/> (b) Human tissues         </div> <div> <input checked="" type="checkbox"/> (c) Neither         </div> </div>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <div style="border: 1px solid black; padding: 10px; min-height: 300px;"> <p>During the past year the reductive activation of the valyl-tRNA synthetase studied was found to be reversible. The enzyme has been observed to oscillate between an active and an inactive form, and the oscillation is affected by several ligands. The implication of this finding is that the reductive process, which regulates the synthetase, is itself regulated, and a cascade of reactions must thus operate to control the rate of the first step of protein biosynthesis.</p> </div>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 23,330-11 LBP
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Aldoheptose Biosynthesis and Its Regulation and Hepatitis Non-A, Non-B		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: William G. Coleman, Jr. Ph.D. Research Microbiologist		LBP NIDDK
Others: Alfred W. Gordon, Ph.D. IRTA Lishi Chen, Ph.D. Visiting Fellow Li Ding, M.D. Special Volunteer Joyce C. Pegues, Ph.D. Special Volunteer		LBP NIDDK LBP NIDDK LBP NIDDK LBP NIDDK
COOPERATING UNITS (if any)  Belinda P. Seto, Ph.D., HL, DBBP, Center for Biologics Evaluation and Research		
LAB/BRANCH Laboratory of Biochemical Pharmacology		
SECTION Section on Pharmacology		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:  4.0	PROFESSIONAL:  3.8	OTHER:  0.2
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.) <p>             Aldoheptose Biosynthesis. An <i>E. coli</i> K-12 strain carrying a <i>cysE-pyrE</i> linked mutation, designated <i>rfaD</i>, which affects the synthesis of L-glycero-D-mannoheptose was isolated and genetically characterized. The <i>rfaD</i> phenotype includes, in addition to altered LPS synthesis, increased permeability to a large number of hydrophobic antibiotics. The <i>rfaD</i> gene initially identified in the Clarke-Carbon Colony Bank was cloned in pBR322, and subsequently smaller restriction fragments were cloned into several plasmid vectors. <i>RfaD</i><sup>+</sup> plasmids express a protein with a molecular weight of 37,000. The protein, ADP-L-glycero-D-mannoheptose-6-epimerase, has been purified to homogeneity and partially characterized. The entire <i>rfaD</i> gene is located on a 1.3 kilobase <i>SspI</i>-<i>HpaI</i> fragment. This region and flanking regions have been completely sequenced, and the coding region and transcription start site have been determined.           </p> <p>             Hepatitis Non-A, Non-B. Hepatitis non-A, non-B (HNANB) is a world-wide problem, and 90% of the transfusion-related hepatitis cases in the United States are diagnosed as HNANB. Approximately 50% of all acute HNANB patients develop chronic HNANB (an estimate of 4 million persons). They remain as potential sources of infection. Recent studies suggest a link between certain hepatocellular carcinomas and chronic HNANB infections.           </p> <p>             Based on biochemical, immunological, and morphological evidence, we suggested that the HNANB agent is a mammalian type C retrovirus or retrovirus-like. Recently, using an <i>in vitro</i> focus-induction assay developed for mammalian type C viruses, we observed that pelleted material from HNANB sera induced foci formation. This result is consistent with the presence of a mammalian type C virus in HNANB sera, and this virus (and the mink lung pseudotype) is the object of isolation and characterization studies.           </p> <p>             A DNA probe of 757 base pairs isolated from HNANB-infected chimpanzee liver was shown to hybridize <i>in situ</i> with liver sections from three HNANB-infected chimpanzees but not with liver from two HBV-infected animals. The probe's specificity for non-A, non-B sequences or for specific HNANB antibodies is under study.           </p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 23,580-26 LBP
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Mammalian Transposons		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Anthony V. Furano, M.D. Medical Officer (Research) and Chief, Section on Genomic Structure and Function, LBP LBP NIDDK	
Others:	Karen Usdin, Ph.D. Visiting Associate LBP NIDDK Bruce E. Hayward, Ph.D. Visiting Fellow LBP NIDDK Esterina Pascale, Ph.D. Visiting Fellow LBP NIDDK Eulalia Valle, Ph.D. Visiting Fellow LBP NIDDK	
COOPERATING UNITS (if any)  None		
LAB/BRANCH Laboratory of Biochemical Pharmacology		
SECTION Section on Genomic Structure and Function		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.5	5.0	0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided )		
<p>Members of the L1 transposon family (long interspersed repeat DNA or LINE family) of rats are 6.7 kb long, 5 kb of which is devoted to protein encoding sequence (ORFs). A very strong promoter is at the left end of the element, and a guanine-rich polypurine:polypyrimidine sequence is near the right end. Although the protein encoding sequences of mammalian L1 families are highly conserved, the promoter sequences are completely distinct. This means that novel species-specific promoter sequences have been repeatedly acquired by L1 families during mammalian evolution and that these families have been reamplified repeatedly in individual mammalian species. Our recent isolation of an L1 element that is ancestral to both the present day rat and mouse L1 families and is as highly repeated in both genomes support this scenario. Our previous demonstration that the rat L1 family contains a very active promoter sequence was the first evidence that L1 DNA is not just some non-functional "junk" DNA. We have further characterized this promoter and shown that the major transcripts most likely begin about 300 bp from the 5' end of the sequence, that the 650 bp promoter consists of both inhibitory and stimulatory modules which can form numerous specific DNA:protein complexes with nuclear extracts as judged by gel retardation experiments. In addition, the intact L1 promoter exhibits a very strong (&gt; 20-fold) synergy with the SV40 promoter sequence. We previously showed that the guanine-rich polypurine:polypyrimidine tract at the right end of an L1 element destabilizes contiguous duplex DNA. We now find that this tract adopts several non-B DNA structures <i>in vitro</i> that may explain this phenomenon. We also found that three randomly chosen L1 insertion sites adopt abnormal DNA structures as well which might facilitate targeting of L1 elements to these regions. In addition the L1 and target site non-B structures compete for supercoil energy which <i>in vivo</i> might modulate the supercoil dependent properties of L1 elements and their target sites.</p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 23,750-03 LBP
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Bacteriophage T4 Gene Expression		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Deborah M. Hinton, Ph.D.	Research Chemist LBP NIDDK
Other:	Richard L. Ellis	Special Volunteer LBP NIDDK
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Biochemical Pharmacology		
SECTION Section on Nucleic Acid Biochemistry		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.6	1.4	0.2
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>During infection, bacteriophage T4 programs the temporal expression of early, middle, and late genes. To examine how T4 regulates middle RNA, we are focusing on the expression of a cluster of T4 prereplicative genes located 5' → 3': X.1 (unknown function, see below), <i>uvvX</i> (recombination protein), 40 (stimulates head formation), and 41 (primase-helicase component).</p> <p>X.1-<i>uvvX</i>-40-41 transcripts are polycistronic, middle RNAs whose major 5' and 3' ends suggest phage-regulation at the level of transcription initiation and elongation or processing. Major 5' ends, 200 and 900 bases upstream of <i>uvvX</i>, show homology with transcription initiation sites dependent on the T4 protein <i>motA</i>. The major 3' end maps just after <i>uvvX</i>, immediately downstream of a GC-rich hairpin. This end apparently arises from factor-dependent transcription termination or processing since it is observed in RNA expressed by a X.1-<i>uvvX</i>-40-41 plasmid <i>in vivo</i>, but is not seen after transcription with RNA polymerase <i>in vitro</i>.</p> <p>In the <i>E. coli</i> transcription termination mutant <i>rho026</i>, termination is not effectively overcome by phage lambda antitermination. During a T4/<i>rho026</i> infection, the level of 41 protein is depressed; a T4 mutant in <i>goF</i> gives a wild type amount of 41. Our RNA analyses demonstrate that the decrease in 41 protein is accompanied by a similar decrease in 41 RNA. This is due in part to more of the upstream <i>uvvX</i> RNA ending at the mapped 3' end. In a T4 <i>goF1/rho026</i> infection, the relative amount of RNA reading into 41 versus that stopped is close to wild type. These results are consistent with a termination or processing site located downstream of <i>uvvX</i> whose use involves <i>rho</i> and <i>goF</i>.</p> <p>Upstream of <i>uvvX</i> lies 840 bp of DNA which is genetically unmapped, but is co-expressed with <i>uvvX</i>, 40 and 41. Our sequencing reveals a 221 amino acid open reading frame, designated X.1, which shares a region of similarity with 2 other T4 genes: gene 69 (putative replication protein) and the ORF 5 (unknown function) upstream of tRNA Arg. <i>In vitro</i> transcription/translation of plasmids and restriction fragments including the X.1 region yields a protein of ~25,000 daltons, consistent with expression of the X.1 protein.</p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 24,140-23 LBP
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <u>Tryptophan Synthase: Structure and Function and Relationship to Tryptophanase</u>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: Edith Wilson Miles, Ph.D.	Research Chemist	LBP NIDDK
Others: Hiroshi Kanzaki, Ph.D. Arvind M. Kayastha Yoshihiro Sawa, Ph.D. Osao Adachi, Ph.D. Hidehiko Kumagai, Ph.D.	Visiting Fellow Visiting Fellow Visiting Fellow Special Volunteer Special Volunteer	LBP NIDDK LBP NIDDK LBP NIDDK LBP NIDDK LBP NIDDK
COOPERATING UNITS (if any) Drs.D.R.Davies, C.C.Hyde, and E.A.Padlan, LMB, NIDDK; P.McPhie, LBM, NIDDK; M.Roy and M.F.Dunn, Univ. of California,Riverside; C.R.Matthews, Pennsylvania State Univ., University Park; A. Mozzarelli and G.L.Rossi, Univ. of Parma, Italy; K. Yutani, Osaka Univ., Japan		
LAB/BRANCH Laboratory of Biochemical Pharmacology		
SECTION Section on Pharmacology		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS  4.0	PROFESSIONAL:  3.8	OTHER:  0.2
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  Our studies of the tryptophan synthase $\alpha_2\beta_2$ complex are aimed at relating enzyme structure to enzyme function. The tryptophan synthase multienzyme complex is an excellent model system for investigating enzyme mechanism, protein-protein interaction, metabolite channeling, and ligand-dependent site-site interactions. We have used x-ray crystallography to determine the three-dimensional structure of the tryptophan synthase $\alpha_2\beta_2$ complex from <i>Salmonella typhimurium</i> . A striking finding is the presence of a tunnel linking the two active sites and permitting the diffusion of indole from the active site of the $\alpha$ subunit to the active site of the $\beta$ subunit. Microspectrophotometric studies on single crystals of the complex demonstrate that chromophoric intermediates are formed between pyridoxal phosphate and substrates at the active site of the $\beta$ subunit and that ligand-dependent site-site interactions occur in the crystal. We are using rapid-scanning stopped-flow spectroscopy to detect the formation and decay of transient intermediates in the reactions of wild type and mutant forms of tryptophan synthase with substrates and with reaction intermediate analogs. We use site-directed mutagenesis to investigate the effects of single amino acid replacements on enzyme activity and on the channeling of reaction intermediates. Our results provide evidence for catalytic roles for glutamic acid 49 and aspartic acid 60 in the active site of the $\alpha$ subunit and for lysine 87 and glutamic acid residues 109 and 350 in the active site of the $\beta$ subunit. Mutant forms of tryptophan synthase are being further characterized by x-ray crystallography, calorimetry, and circular dichroism measurements.		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		<b>PROJECT NUMBER</b>  Z01 DK 24,150-18 LBP
<b>PERIOD COVERED</b> October 1, 1988 to September 30, 1989		
<b>TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)</b> Noncovalent Intermolecular Interactions in Biochemistry		
<b>PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)</b>		
<b>PI:</b> Allen P. Minton, Ph.D.	Research Chemist	LBP NIDDK
<b>Others:</b> Susan Lakatos, Ph.D. Shu-Hui L. Hsu, Ph.D.	Visiting Associate IRTA Fellow	LBP NIDDK LBP NIDDK
<b>COOPERATING UNITS (if any)</b> M. S. Lewis, Biomedical Engineering and Instrumentation Branch, Division of Research Services, NIH; R. C. Chatelier, Commonwealth Scientific and Industrial Research Organization, Melbourne, Australia		
<b>LAB/BRANCH</b> Laboratory of Biochemical Pharmacology		
<b>SECTION</b> Section on Pharmacology		
<b>INSTITUTE AND LOCATION</b> NIDDK, NIH, Bethesda, Maryland 20892		
<b>TOTAL MAN-YEARS:</b>  2.5	<b>PROFESSIONAL:</b>  2.3	<b>OTHER:</b>  0.2
<b>CHECK APPROPRIATE BOX(ES)</b> <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
<b>SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)</b>  <p>The interaction between fibrous rabbit muscle actin and the globular proteins aldolase and bovine serum albumin has been studied by sedimentation equilibrium and electron microscopy.</p> <p>The PC-based data acquisition and control system for the analytical ultracentrifuge, which was initially developed and installed last year, has been substantially upgraded with new hardware and extensively revised software.</p> <p>The ultracentrifuge simulation program has been extended to treat the case of concentrated macrosolutes, the sedimentation of which cannot be validly described in the ideal approximation.</p> <p>Models for estimation of the fraction of cytoplasmic volume that may be considered adjacent either to boundary membranes or to the surface of fibrous elements comprising the cytomatrix have been formulated.</p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 24,260-23 LBP
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Enzymatic Mechanisms of DNA Replication: The Bacteriophage T4 System		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: Nancy G. Nossal, Ph.D. and Chief, Section on Nucleic Acid Biochemistry. LBP	Research Chemist	LBP NIDDK
Others: Peter Spacciapoli, Ph.D. Richard L. Ellis, B.A. Helen C. Hollingsworth, M.D.	IRTA Special Volunteer Special Volunteer	LBP NIDDK LBP NIDDK LBP NIDDK
COOPERATING UNITS (if any)  None		
LAB/BRANCH Laboratory of Biochemical Pharmacology		
SECTION Section on Nucleic Acid Biochemistry		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 208		
TOTAL MAN-YEARS  2.7	PROFESSIONAL:  2.5	OTHER:  0.2
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  We are continuing our study of the <u>E. coli</u> bacteriophage T4 model system for duplex DNA replication in which efficient DNA replication <u>in vitro</u> is achieved with seven purified proteins encoded by T4 phage: T4 DNA polymerase (gene 43), gene 32 DNA helix-destabilizing protein, the gene 44/62 and gene 45 polymerase accessory proteins, and the genes 41 and 61 primase-helicase. <i>Interactions between the T4 Replication Proteins.</i> Using synthetic model templates, we have shown that the T4 replication enzymes cannot displace a completely complementary DNA, and thus are able to avoid displacing the previous discontinuous fragment on the lagging strand. In contrast, a fork as short as 50 bases allows continuous strand displacement synthesis on the leading strand. The initiation of new fragments on the lagging strand decreases when either the gene 61 primase protein or DNA polymerase is limited. However, once lagging strand synthesis is established, dilution experiments indicate that 61 protein and polymerase remain bound to the lagging strand template to a significant extent during successive rounds of synthesis of Okazaki fragments. <i>Ribonuclease H in T4 Infected Cells Removes RNA Primers on the Lagging Strand.</i> An RNase H activity increasing after T4 infection has been extensively purified. This enzyme can completely remove the pentamer RNA primers from DNA made by the T4 replication proteins <u>in vitro</u> . Addition of the T4 RNase H and T4 DNA ligase to the seven other replication proteins leads to extensive joining of adjacent discontinuous fragments, indicating that these enzymes are sufficient for primer release, gap filling, and DNA chain sealing on the lagging strand. The N-terminus of a protein copurifying with the RNase H matches that encoded by a predicted open reading frame in the T4 genome. The DNA containing this open reading frame is being cloned to determine if it encodes the RNase H activity. <i>Essential Regions of T4 DNA Polymerase.</i> T4 DNA polymerase has six regions which share sequence similarity with several eukaryotic DNA polymerases. To determine the function of shared and unique regions, we are characterizing altered T4 DNA polymerases produced by site-specific mutagenesis of the cloned gene.		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 24,590-18 LBP
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Structure and Interactions of Biologically Important Macromolecules		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: Harry A. Saroff, Ph.D. Research Chemist (Intermittent)		LBP NIDDK
Other: Elemer Mihalyi, Ph.D., Ph.D. Special Volunteer		LBP NIDDK
COOPERATING UNITS (if any) A. Patchornik, Weizmann Institute of Science, Rehovot, Israel; National Center for Drugs and Biologics		
LAB/BRANCH Laboratory of Biochemical Pharmacology		
SECTION Section on Pharmacology		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 1.8	PROFESSIONAL: 1.6	OTHER: 0.2
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)  Cooperative binding systems are being studied taking into account site or sub-unit interactions, ligand interactions, aggregation and redistribution in proteins, and model systems. Methods are being developed to evaluate reasonable values for the parameters describing these systems. Changes in calcium binding to fibrinogen upon clotting by thrombin were investigated and correlated with the release of fibrinopeptides and the polymerization steps.  Amino acid sequences of proteins are analyzed primarily with the Monte Carlo techniques to evaluate the uniqueness and homology of these sequences. The property of uniqueness (the occurrence of a <i>small</i> peptide at a frequency considerably less than that expected) has been quantified, and speculations on this quantity and the immune response are under investigation.		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 24,709-08 LBP
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Polyamine Biosynthesis and Function		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: Celia White Tabor, M.D. Medical Officer (Research) LBP NIDDK  Others: Herbert Tabor, M.D. Supervisory Medical Officer (Research); Chief, Section on Pharmacology, LBP; and Chief, Laboratory of Biochemical Pharmacology LBP NIDDK Qiao-Wen Xie, Ph.D. Visiting Associate LBP NIDDK David Balasundaram, Ph.D. Visiting Fellow LBP NIDDK Keiko Kashiwagi, Ph.D. Visiting Fellow LBP NIDDK		
COOPERATING UNITS (if any) D. T. Liu and N. Y. Nguyen, Division of Biochemistry and Biophysics, CBER; K. W. Minton, Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, Maryland		
LAB/BRANCH Laboratory of Biochemical Pharmacology		
SECTION Section on Pharmacology		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 5.2	PROFESSIONAL: 3.7	OTHER: 1.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  Polyamines (putrescine, spermidine, and spermine) are major cellular components, and have been shown to be involved in many systems related to growth and differentiation. Our studies have been directed at learning how these polyamines are synthesized and regulated, and their physiological function. We have: (1) established the pathways for the biosynthesis of these amines; (2) isolated the enzymes involved; (3) identified the genes responsible for each of these steps and constructed mutants lacking the encoded enzymes; (4) constructed plasmids that contain these genes, and that permit overproduction of the various enzymes; (5) studied the physiological effects of amine deprivation <i>in vivo</i> on ribosome action and on protein biosynthesis; (6) shown that the genes coding for spermidine synthase ( <i>speE</i> ) and for <i>S</i> -adenosylmethionine decarboxylase ( <i>speD</i> ) form an operon at 2.7 minutes on the <i>Escherichia coli</i> chromosome; we have sequenced and characterized this operon and shown that this operon extends 514 bp above the ATG start site for <i>speE</i> ; the upstream part of the operon includes several upstream promoters and an additional upstream open reading frame of unknown function; (7) shown that <i>S</i> -adenosylmethionine decarboxylase is formed as proenzyme which is then processed by a post-translational cleavage at a lysyl-serine peptide to form two subunits, one of which contains the pyruvoyl group that is found in the mature enzyme and is essential for enzymatic activity. Mutants in which other amino acids are substituted for the lysine result in much slower processing. (8) We are purifying the intact proenzyme and are studying the cleavage mechanism. (9) We have carried out comparable studies on the gene for <i>S</i> -adenosylmethionine decarboxylase ( <i>SPE2</i> ) in <i>Saccharomyces cerevisiae</i> . We have completed the cloning and sequencing of the <i>SPE2</i> gene, overproduced the encoded protein in <i>E. coli</i> , and have obtained large amounts of pure enzyme. (10) This protein (from <i>S. cerevisiae</i> ) is also synthesized as a proenzyme; the mature enzyme is formed by a specific cleavage at a glutamic acid-serine junction, into a 10,000 Mr and a 36,000 Mr fragment that contains a pyruvate at the new amino terminal end.		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 24,940-16 LBP
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Yeast RNA Virology		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: Reed B. Wickner, M.D. Medical Director, USPHS and Chief, Section on Genetics of Simple Eukaryotes, LBP LBP NIDDK Others: Tsutomu Fujimura, Ph.D. Visiting Scientist LBP NIDDK Jonathan D. Dinman, Ph.D. IRTA LBP NIDDK Yutaka Matsumoto, Ph.D. IRTA LBP NIDDK Tateo Ichio, Ph.D. Visiting Associate LBP NIDDK Hyun-Sook Lee, Ph.D. Special Volunteer LBP NIDDK Yang-Ja Lee, Ph.D. Special Volunteer LBP NIDDK Juan C. T. Lopez, Ph.D. Special Volunteer LBP NIDDK		
COOPERATING UNITS (if any)  None		
LAB/BRANCH Laboratory of Biochemical Pharmacology		
SECTION Section on Genetics of Simple Eukaryotes		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 5.5	PROFESSIONAL: 5.1	OTHER: 0.4
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>           There are five families of double-stranded RNA (dsRNA) that replicate in cells of the yeast <i>Saccharomyces cerevisiae</i>. Three of these, L-A, L-BC, and M, replicate in viral particles. A single-stranded RNA, called 20S RNA, also replicates in yeast. We have developed an <u>in vitro</u> L-A RNA replication system and determined the essential sites on L-A (+) strands for this reaction. They are the 3' end 4 bases, a stem-loop structure adjacent to these 4 bases, and an internal site (the Internal Replication Enhancer or IRE). Overlapping with the IRE is a site needed for binding to viral particles of (+) single strands (Viral Binding Site or VBS). The VBS includes a stem-loop structure with an A residue protruding on the 5' side. We have developed an <u>in vitro</u> transcription system [(+) strand synthesis from dsRNA template] which we can use to study the template requirements of this reaction as well. This system consists of opened empty viral particles and carries out the conservative, template-specific synthesis of viral (+) strands. This is the only dsRNA virus for which template-dependent <u>in vitro</u> systems have been developed. L-A encodes a fusion protein whose N-terminus is a major coat protein monomer and whose C-terminus has homology with the RNA-dependent RNA polymerases of (+) strand RNA viruses. We have evidence that this protein is formed by ribosomal frameshifting. We have cloned the chromosomal MAK10 genes needed by the L-A virus for its replication, and are studying its structure and regulation. We have isolated a clone of a chromosomal gene that when present on a high copy vector makes <i>ski</i><sup>-</sup> strains no longer grow slowly, that is, these strains are no longer sensitive to the viral pathogenicity otherwise associated with derepression of viral replication that results from a <i>ski</i> mutation. We have isolated clones of most of the 20S ssRNA replicon and obtained evidence that it is a circular RNA replicon, like the human hepatitis delta virus.         </p>		

ANNUAL REPORTS OF THE LABORATORY OF CHEMICAL BIOLOGY  
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The Laboratory of Chemical Biology conducts research on structure, function and dynamics of proteins and on molecular biology and genetics, especially as related to genetic diseases. A major emphasis of the Laboratory is the identification of cis-acting regions in the DNA close to various human globin genes that may be involved in transcriptional control and the identification of the trans-acting proteins which bind to these DNA sequences and effect functional changes. A second major emphasis of the Laboratory is in the study of forces that stabilize globular proteins and also that contribute to the interaction of these proteins and antibodies. A program on isolating and characterizing the tat protein of the HIV virus has also been initiated under sponsorship of the NIH Intramural AIDS Research Program which is specifically targeted to drug development. Other active projects include the clinical evaluation of sickle cell patients being treated with hydroxyurea to increase fetal hemoglobin levels, the study of the erythropoietin receptor and cytogenetic studies of a variety of human disease syndromes.

The Laboratory has three Sections. The Section of Protein Chemistry and Conformation, under Dr. Hiroshi Taniuchi, is devoted primarily to studying the folding and assembly of globular proteins, especially cytochrome c. The Section on Molecular Forces and Assembly is the home of the Cytogenetics Unit under Dr. Beverly White, which is a joint endeavor of the Inter-Institute Genetics Program of the Clinical Center and NIDDK. The Section on Molecular Biology and Genetics, under Dr. Alan N. Schechter, is concerned primarily with the molecular basis of the developmental control of gene expression, especially in human erythroid cells, and its relevance to the understanding of the molecular basis of disease states and possible approaches to their therapy. Among new joint programs with other institutes in the Laboratory, in addition to the Cytogenetics Unit, and the AIDS project, studies of erythropoietin, and its receptor and therapeutic protocols is sickle cell disease. These are detailed below.

During the last year there have been a few major personnel changes. Dr. Beverly White's transfer to this Laboratory to establish a Cytogenetic Unit has been completed. Dr. Griffin Rodgers has completed his four years as a Robert Wood Johnson Fellow and training at George Washington University in Hematology and Oncology, and has returned to this Laboratory as a Senior Medical Staff Fellow. Dr. David Cohen has transferred to NIAID as an Expert Consultant to work on AIDS. Dr. C. B. Anfinsen continues as a Scientist Emeritus in this Laboratory and makes frequent visits here.

Extensive research collaborations exist within this Laboratory and with other Laboratories in this Institute, in NIH, and nationally and internationally as outlined in the individual Research Project Reports. A formal collaboration has been established with the Clinical Center's Inter-Institute Medical Genetics Program to fund a clinical and research cytogenetic program. Clinical collaborations also exist with the Clinical Hematology Branch of NHLBI and other units. It is under the aegis of this collaboration that the clinical studies of hydroxyurea treatment of sickle cell patients is done. Development of transgenic sickle cell mice is being done in collaboration with the Metabolic and Developmental Neurology Branch of NINCDS. In addition, a formal collaboration has been established involving the exchange of personnel and resources with Dr. David Hankins of the Laboratory of Experimental Hematology of the Armed Forces Radiobiological Research Institute at the National Naval Medical Center and the Division of Hematology of the Children's National Medical Center. The participation of this Laboratory in the NIH Inter-Institute Medical Genetics Program and the NIH-George Washington University Hematology Training Program continues to grow. The Laboratory is now also a major part of the recently established Intramural AIDS Research Program. The work in this program involves collaborations with the Laboratory of Molecular Virology of NCI and KabiGen AB of Stockholm, Sweden. Other collaborations include those with the Inserm Unit at Hôpital Henri Mondor at Creteil, France (including an exchange of personnel); the Division of Hematology at the University of Birmingham School of Medicine in England and the MRC Unit in Kingston, Jamaica.

### Section on Protein Chemistry and Conformation

A new hypothesis for protein folding has been developed during the last several years. This is based on the studies of fragment complexes of cytochrome *c*, in particular the stability of various heterologous complexes and ones chemically synthesized with changed amino acids and of the binding of various monoclonal antibodies to cytochrome *c*. Originally these studies were interpreted generally in terms of globally coupling forces. But now they are more specifically being studied as a manifestation of a small number of closed loops for each protein that mediate the stability of the folded forms. For cytochrome *c* it is postulated that there are four such loops controlling stability. Analogously, the extreme sensitivity of antigen-antibody interactions to single amino acid changes such as a change in binding constant of 10,000 following a substitution of glutamic acid 93 by alanine in cytochrome *c*, is also explained in terms of interatomic interactions of the closed loop - type between the protein and the antibody. This analysis of protein folding and stability is a new way of explaining perplexing problems remaining in more classical approaches.

Among the techniques being used in these studies and fragment complex exchange measurements; chemical synthesis; and production and characterization of monoclonal antibodies. A new state of the art amino acid analyzer has added a major instrument resource to the work of this Section.

### Section on Molecular Forces and Assembly (Cytogenetics Unit)

This Unit has completed cytogenetic analyses of almost 400 patients participating in NIH clinical protocols and analyzed their results. The study of the correlation between nucleolus organizing regions (NOR) and Alzheimers disease has been completed with largely negative results, as has been the case for an examination of a possible correlation between schizophrenia and the fragile X chromosome syndrome. Several other studies are underway including an analysis of embryonic stem cells for the development of transgenic mice..

### Section on Molecular Biology and Genetics

The major part of this Section's work is devoted to clarifying the molecular genetic basis by which the developmental switch from embryonic to fetal to adult hemoglobins occurs in the human. Understanding of the control of globin gene expression would be a very important general point with respect to developmental biology, but might also have specific therapeutic relevance for the diseases of hemoglobin. The project is being pursued for the most part by trying to understand the phenotype of a cell line, the K562 cells, which appears to be arrested in the late embryonic stage of globin gene expression. Evidence has been obtained that there are intranuclear factors, *trans*-acting factors, that determine which genes are expressed and which are silent in these cells. During the last year, a broad range program to identify and isolate these factors and to understand their mechanism of action has been developed. To this end studies are underway of the structure and function of the globin promoter regions (*cis*-acting sequences) by fusing families of deletion mutants to the gene for the enzyme chloramphenicol transferase (CAT) and assaying CAT activity in cells transfected with various promoter-CAT fusion genes. In addition direct binding assays (footprinting and gel shift) and subtractive cloning techniques are being used in order to isolate the protein or the gene for one or more of these *trans*-acting factors. During the last year these techniques have been used to identify several *cis*-sequences and several proteins (*trans*-acting) factors that interact in the 5' region of the human  $\beta$ - and  $\epsilon$ -globin gene. The functional significance of each of these is now being investigated. In addition an *in vitro* transcription system has been developed which is specific for different globin genes and several genes have been cloned from induced K562 cells which may be involved in the induction phenomenon. Although the goals of characterizing these factors are not simple, the elucidation of the control of this biologically and medically important human gene system would be a potentially major step in molecular and development biology and in applied medical molecular genetics.

This Section also continues its work on the pathophysiology of sickle cell anemia. During the last year the role of fetal hemoglobin levels in determining disease severity and expected response to therapy has been clarified. A project to develop an animal model of sickle cell disease by using embryonal carcinoma

cells and transgenic methods to introduce the  $\beta^S$  and the human  $\alpha$  gene into mice has been initiated. Methods to remove the endogenous mouse globins, including site specific recombination, are also being studied. This work is regarded as a long term project to develop a true model of the disease for study of sickle cell rheology, pathophysiology, and treatment. A Clinical Center program to treat select patients with hydroxyurea has been initiated and ten patients have been treated with significant elevations in fetal hemoglobin levels. Modified protocols using erythropoietin as well as hydroxyurea has been initiated.

The NIH Intramural Research Program on AIDS has established a program in this Section to analyze transcriptional mechanisms related to the *tat* gene of HIV. The cloned *tat* gene has been obtained and has been inserted into an expression vector for large scale production of the *tat* protein in *E. coli* and eukaryotic cells. Several batches of the protein have been produced and partially purified from *E. coli* by KabiGen of Stockholm, Sweden and are now being further purified and characterized in this laboratory as is material made in insect cells with a baculo virus vector. The protein will be purified to allow detailed structure function studies, including x-ray crystallography and high resolution NMR. The *tat* gene is also being transfected into heterologous cells to examine its interaction with other promoters so as to clarify its molecular mechanism of action. We are trying to develop structural (binding) and functional assays to allow systematic study of potential inhibitors of *tat* function. We hope these studies may lead to a new approach to the treatment of AIDS.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25008-26 LCB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Closed Interaction Loops That Control Protein Folding and Function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Hiroshi Taniuchi Chief, Section on Protein LCB, NIDDK  
Chemistry and Conformation

Other: Alice Fisher Chemist LCB, NIDDK  
Xuan Truong Biological Aid LCB, NIDDK  
Greg A. Charles Biological Aid LCB, NIDDK

## COOPERATING UNITS (if any)

University of Padova, Padova, Italy

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Protein Chemistry and Conformation

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

2.1

## PROFESSIONAL:

1.6

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The polypeptide chains of proteins are assembled by the cellular mechanism on the basis of the information stored in the linear sequence of DNA. This genetic code is known. However, the mechanism by which the assembled polypeptide chain folds to the three-dimensional structure with function, that is, the second half of the genetic code, is largely unknown and a subject of intensive studies in many laboratories. We have taken an approach to cracking this second half of the genetic code by studying the cooperative interaction, the fundamental property of protein. Thus, to dissect this cooperative interaction we have developed the fragment complementing systems of staphylococcal nuclease and cytochrome c. Our studies of these systems and RNase A derivatives in the previous years have suggested that (1) a previously unknown non-covalent interaction may exist in proteins, (2) this interaction is exothermic and has a delocalized nature, and (3) this interaction is sensitive to the change of configuration or local order of the groups involved and, therefore, has the ability to order the polypeptide chain. This has led us to the hypothesis that the new delocalized interaction is mediated by contacting groups which form a closed loop in the interior. To locate such closed interaction loops with cytochrome c, we have determined the dissociation constants of the homologous and hybrid complexes formed from the heme and apofragments or apoproteins of horse, tuna, yeast iso-1 and Candida cytochrome c. The homologous and hybrid complexes were prepared with respect to three different types of complexes containing the discontinuity of the polypeptide chain at three different regions. The results show that depending on which species of heme or apofragment is used or which combination of heme and apofragment is used, the complex exhibits distinctly different magnitudes of the dissociation constants. The results are consistent for all three types of complexes. Comparing the amino acid sequences, these data have allowed us to assign five different mutations related to the observed differences of dissociation constant. The mutations thus assigned are located in the specific region which includes the right hydrophobic channel found by R. E. Dickerson and colleagues. Furthermore, perturbation due to these mutations is difficult to explain on the basis of hydrophobic interaction, van der Waals interaction, hydrogen bonds and electrostatics. Thus, we propose that the new delocalized interaction exists in this hydrophobic core region and provides the major force for folding of cytochrome c.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 25011-15 LCB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Principles That Govern Protein Binding: Delocalized Non-covalent Interaction		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Hiroshi Taniuchi  Others: Boleslaw Picur	Chief, Section on Protein Chemistry and Conformation  Visiting Fellow  LCB, NIDDK  LCB, NIDDK
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Chemical Biology		
SECTION Section on Protein Chemistry and Conformation		
INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland		
TOTAL MAN-YEARS:  <div style="text-align: center;">0.9</div>	PROFESSIONAL:  <div style="text-align: center;">0.9</div>	OTHER:  <div style="text-align: center;">0</div>
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  As described in another report, our studies of protein folding in the previous years have led us to the hypothesis that (1) a new non-covalent interaction of a delocalized nature exists in proteins; (2) this interaction is exothermic and mediated by contacting groups which forms a closed loop in the interior; and (3) this interaction is sensitive to change of the configuration or local order of the groups involved and, therefore, has the ability to order the polypeptide chain. To obtain evidence for such delocalized interaction, we have studied perturbation of the energy barrier of unfolding by amino acid substitution. For this, we have used three-fragment complex of horse cytochrome c consisting of heme fragment of residues 1 to 25, (1-25)H and apofragments (28-38) and (39-104). Leucine (L) 32 or 35 of fragment (28-38) was substituted with norvaline (norV) by chemical synthesis. Both leucines are buried in the interior and contribute to the heme environment (R. E. Dickerson et al.). We have shown previously that dissociation of fragment (39-104) from three-fragment complex ferro (1-25)H:(28-38):(39-104) can occur without going through two fragment complex (1-25)H:(39-104). We assume that this type of dissociation represents a major aspect of unfolding of the native protein. The rate of this dissociation was determined as a function of temperature by the fragment exchange technique developed in the previous years. The values for activation enthalpy thus found are 33.8, 21.9, 19.1 and 9 kcal/mol, respectively for the complexes with none, L32 to norV and L35 to norV substitutions and that containing norV at both positions 32 and 35. It was found previously that the magnitude of negative enthalpy change associated with binding of fragment (28-38) decreases by 12.9, 22.5 and 25.8 kcal/mol respectively by L32 to norV, proline 30 to glycine and glycine 34 to alanine substitutions. These results, taken together with the fact that leucine 32 does not contact residues 39 to 104 (R. E. Dickerson et al.) suggest the following: (a) Perturbation of activation free energy by L32 to norV and L35 to norV substitution is non-additive; (b) L32 to norV substitution mainly perturbs the delocalized interaction; and (c) the source of activation enthalpy is due mainly to disruption of the delocalized interaction instead of cleavage of many hydrogen bonds which is generally assumed to be the source for unfolding of proteins. Thus, the existence of the new delocalized interaction binding the polypeptide chain in the ordered state appears to be supported.		

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25016-16 LCB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Trans-Acting Factor(s) Controlling Globin Gene Expression in K562 Cells

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Alan N. Schechter	Chief	LCB, NIDDK
Others:	Shi-Xian Cao	Visiting Associate	LCB, NIDDK
	Harish Dave	Visiting Fellow	LCB, NIDDK
	Pablo Gutman	Visiting Fellow	LCB, NIDDK

## COOPERATING UNITS (If any)

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

1.1

## PROFESSIONAL:

1.1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

K562 is an erythroleukemic cell line used as a model for the study of the control of human globin gene expression. These cells do not support transcription of the beta-globin gene (human adult pattern of expression) but do express transcripts of epsilon- and gamma-globin genes (human embryonic and fetal pattern) at very high levels when exposed to a number of inducing agents. Results from this and other laboratories suggest that the control of this pattern of expression is mediated by the presence and/or absence of trans-acting factors which exert their action on sequences corresponding to the promoters of these genes. Sequence specific DNA binding proteins acting on cis-regulatory elements have been hypothesized to be key elements in eukaryotic gene transcription, and even though considerable progress has been made in their isolation, DNA binding proteins with affinity for the human globin gene promoters have not yet been identified. We have defined several positive and negative regulatory regions 5' to the epsilon-globin promoter, and detected binding of proteins to these regions.

The methodology used included DNase footprinting and the gel retardation assay. One of the negative regions defined binds to a factor present in high concentrations in non-erythroid cells. Using in vitro mutagenesis, inhibition of binding of a protein to this region causes a 10 fold activation of the epsilon-globin gene promoter in a CAT-assay. This technique has also allowed us to further define several positive and negative regulatory sequences 5' to the protein coding region of this gene.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25021-14 LCB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Sickle Cell Anemia: The Intracellular Polymerization of Hemoglobin S

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Constance Tom Noguchi Research Physicist LCB, NIDDK

Others: Alan N. Schechter Chief LCB, NIDDK

## COOPERATING UNITS (if any)

LCDB, NIDDK (J. Blanchette-Mackie); NINCDS (Dr. S. Karlsson), Birmingham, U.K.  
(Dr. J. Stuart), Johns Hopkins (Dr. S. Charache), University of Calif.,  
San Francisco (Dr. N. Mohandas), Sydney Hospital, Australia (Dr. E. Raik).

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

0.5

## PROFESSIONAL:

0.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Hemoglobin S polymerization within intact erythrocytes is determined primarily by oxygen saturation, hemoglobin concentration and hemoglobin composition. We have examined the filterability of sickle erythrocytes using an initial flow-rate method and demonstrated the impairment of erythrocyte filterability at high oxygen saturation. This loss of filterability through 5  $\mu$ m diameter pores occurred at higher  $pO_2$  than did morphological sickling. To examine the extent of polymerization corresponding to the loss of filterability, erythrocytes from homozygous sickle cell anemia (SS) patients were fractionated on a density gradient to obtain more uniform subpopulations of cells. The dense cell or higher corpuscular hemoglobin fraction exhibited loss of filterability at minimal oxygen desaturation with the apparent formation of little or no intracellular polymer. Lighter cell fractions corresponding to lower corpuscular hemoglobin concentration required a greater degree of deoxygenation and polymer formation to impair deformability. When compared with the whole cell or unfractionated population, the dense cells in sickle cell disease exert a disproportionate effect on blood rheology and are likely to have an adverse rheological effect in vivo at arterial oxygen tension.

Hemoglobin Setif, an  $\alpha$ -chain mutation, exhibits reduced solubility in phosphate buffered saline. When incubated in select buffers, erythrocytes containing hemoglobin Setif pseudosickled and loss deformability. Pseudosickling and loss of deformability were accelerated at higher osmolarities. Hemoglobin lysates containing hemoglobin Setif demonstrated a corresponding reduced solubility which increased when temperature was reduced from 37°C to 40°C. Compared with SS erythrocytes, hemoglobin Setif containing erythrocytes have increased solubility and slower kinetics of aggregation. Detailed studies of hemoglobin Setif aggregation may provide insight into pathophysiology arising from hemoglobin aggregation and may suggest alternate strategies for therapeutic intervention in sickle cell disease.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25025 13 LCB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Specificity and Complement Binding Effect of Antigen-Antibody Interaction

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Hiroshi Taniuchi Chief, Section on Protein LCB, NIDDK  
Chemistry and Conformation

Others: Antonello Punturieri Visiting Fellow LCB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Protein Chemistry and Conformation

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

1.1

## PROFESSIONAL:

1.1

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

As described in another report, our studies of protein folding in the previous years have led us to the hypothesis that (1) the previously unknown, delocalized non-covalent interaction exists in proteins; (2) this interaction is exothermic and mediated by contacting groups which form a closed loop in the interior; and (3) this interaction is sensitive to change of the configuration or local order of the groups involved and, therefore, has the ability to order the polypeptide chain. It is well known that single amino acid substitution of protein or peptide antigens has a profound effect on the affinity to antibody, even if the substitution does not involve a change of hydrophobicity or charge. This phenomenon is strikingly similar to the effect of single amino acid substitution on fragment complexation which is assumed to be a manifestation of the new delocalized interaction. Thus, we wish to investigate if such delocalized interaction is involved in antigen recognition, and the mechanism by which antigen binding influences complement binding. For this end we have prepared 7 monoclonal antibodies recognizing different epitopes of yeast iso-1-cytochrome c or apoprotein in the previous years. Two of these, 2-96-12 (IgG2a) and 4-74-6 (IgG1) were purified using a streptococcal protein G affinity column. Hydrogen-deuterium exchange of the purified monoclonals was studied at pD7.4, 25 degree C in the presence and absence of the antigen, yeast iso-1-cytochrome c, by infrared spectrophotometry. The results show that exchange rates of the amide protons of monoclonal 2-96-12 that exchange after 1.5 h decreases upon binding to the antigen. These affected amide protons represent approximately 5 per cent of the total corresponding to 33 amide protons per antigen binding site. The results with monoclonal 4-74-6 are also similar. These results indicate that the antigen binding alters the energy states of residues of the antibody that are remote from the antigen binding site. This is consistent with the hypothesis that extra force for antigen binding is provided by the delocalized interaction mediated by a closed interaction loop formed within or across the interface between antigen and antibody. This closed loop could involve the residues of antibody which are remote from the interface.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 25028-11 LCB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) The Development of Non-Invasive Methods to Assess Sickle Cell Patients		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Griffin P. Rodgers	Senior Staff Fellow  LCB, NIDDK
Others:	Constance T. Noguchi Alan N. Schechter	Senior Investigator Chief  LCB, NIDDK LCB, NIDDK
COOPERATING UNITS (if any) Clinical Hematology Branch, NHLBI (A.W. Nienhuis); Clinical Branch, NEI (M. Roy); Transfusion Medicine, CC (H. Klein); BEIB (Eli Walker); Biometry Branch, NEI (M. Podgor); MRC Laboratory, Kingston, Jamaica (G. Serjeant).		
LAB/BRANCH Laboratory of Chemical Biology		
SECTION Section on Molecular Biology and Genetics		
INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland		
TOTAL MAN-YEARS 0.3	PROFESSIONAL 0.3	OTHER 0
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>             The molecular and cellular pathophysiology of the sickle cell syndromes are now appreciated with a great deal of precision. On the other hand, our understanding of the relationship of these subcellular events to the variable clinical expression of sickle cell disease remains largely speculative. A major effort of our research group has been to develop quantitative ways to clarify disease pathogenesis, as well as to assess severity and progression. Using calibrated phthalate ester separation method, which we previously described, we have now defined at least three cellular processes contributing to the extensive red cell heterogeneity that is commonly observed in the sickle cell syndromes. Ophthalmological studies of the patients show highly significant correlations between the extend of erythrocyte heterogeneity with conjunctival and retinal vessel pathology. As predicted by biophysical studies of polymer formation, we find that treatment of steady state sickle cell patients with selective arteriolar vasodilators results in a significant resolution of both acute conjunctival and retinal abnormalities, as well as an improvement in color vision performance. These beneficial effects occurred in the absence of a direct drug-induced inhibition of polymer formation, and therefore suggests that inappropriate vasospasm or frank vasoconstriction, perhaps in response to the altered rheology of red cell containing polymerized sickle hemoglobin is a significant contributing factor to the pathogenesis of sickle cell disease. This conclusion is also supported by our recent observation that "relative" hypertension is a significant risk factor for the occurrence of stroke in sickle cell patients. Using the technique of laser-Doppler velocimetry, we have found that forearm cutaneous microcirculatory flow undergoes a unique characteristic periodic pattern, which may become more "normalized" depending upon the fraction of non-S hemoglobins and during crisis. We hope that these cellular and physiological measurements will allow us to understand better the extreme spectrum of disease manifestations.           </p>		

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25038-09 LCB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effects of HTLV-I Tat-I Product on Globin Gene Expression

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Henry B. Fox Staff Fellow LCB, NIDDK

Others: Alan N. Schechter Chief LCB, NIDDK

## COOPERATING UNITS (if any)

Armed Forces Radiobiological Research Institute, Bethesda, MD  
(Drs. W. D. Hankins, and H. F. Fox)

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

0.8

## PROFESSIONAL:

0.8

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The control of human globin gene expression in erythroid cells involves trans-factors (substances active at distant locations in the genome), most of which have yet to be identified or clearly described. One experimental approach to their identification is to study the effects on globin gene expression of well-described trans-factors from tumor viruses. We have shown that the HTLV-I trans-factor tax<sub>1</sub> stimulates both beta- and epsilon-promoters fused to a CAT gene, resulting in roughly 20-fold increase in CAT enzyme activity. In the case of beta-globin, only 185 bp of 5' flanking sequence is required for this effect. Also, we have shown that stimulation of the epsilon-globin promoter requires at least 114 but not more than 177 bp of 5' flanking sequence. Computer analysis reveals 2 elements in this region homologous to the tax<sub>1</sub> response element of HTLV-I; their functional significance has been demonstrated by site directed mutagenesis. In addition, we have demonstrated sequence-specific binding of nuclear proteins from HeLa and K562 cells to one of these elements (-121/-114). Binding requires both the tax<sub>1</sub>-response-like element and an intact CACCC sequence (a cis element important to the activation of many genes) at -111/-107.

Further studies will involve characterization of the tax<sub>1</sub> induced trans-activation of globin promoters. Identification of these cis elements and the proteins that bind to them may lead to identification of proteins involved in trans-activation of globin genes by tax<sub>1</sub>. Our ultimate objective is to identify such cellular proteins that interact with tax<sub>1</sub> to trans-activate globin genes. Study of such proteins may clarify the developmental regulation of globin gene expression in human erythroid cells.

..A

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25045-06 LCB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Globin Gene Expression by 5' Silencer DNA Sequences

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Patricia Berg Senior Staff Fellow LCB, NIDDK  
Others; Alan N. Schechter Chief LCB, NIDDK

## COOPERATING UNITS (if any)

Laboratory of Molecular Hematology, NHLBI (Dr. R. Cohen)

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

1.1

## PROFESSIONAL:

1.1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The regulation of expression of the human globin genes is still poorly understood. Because of its importance in approaches to the therapy of the hemoglobinopathies, understanding regulation of the  $\beta$ -globin gene is the focus of this research. Experiments by others involving hybrid genes in which 5' or 3' positions of  $\beta$ -globin DNA relative to the cap site were fused to heterologous genes have shown that both regions contain regulatory sequences. Recently, published reports have identified nuclear proteins which bind to regulatory DNA located between the cap site and -200 bp. We wanted to determine whether additional regulatory regions are present between -600 and -200 bp and, if so, to identify nuclear proteins which bind to them and may contribute to this regulation.

Our deletion analysis has shown there are at least three regulatory regions 5' to -200 bp of the  $\beta$ -globin gene, two negative regulatory regions and one positive regulatory region. We have identified several proteins which bind to the two negative regions, and the positive region. One protein, which we call BP1, binds to both negative regions as well as to a negative region 5' to the mouse  $\beta$ maJ - globin gene. We believe BP1 may be a repressor protein.

In this regard, we have studied binding of BP1 to a mutant DNA sequence found in a patient who is a silent carrier of  $\beta$ -thalassemia and who exhibits decreased  $\beta$ -chain synthesis. BP1 binds up to nine-fold more strongly to the mutant DNA than normal DNA, again suggesting BP1 may act as a repressor. Further investigation of BP1 and the other binding proteins we have identified is underway.

## DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

PROJECT NUMBER

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25049-05 LCB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Identification of a Silencer Element in the Human Epsilon-Globin Gene

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Shi Xian Cao	Visiting Associate	LCB, NIDDK
Others:	Pablo Gutman	Visiting Fellow	LCB, NIDDK
	Harish Dave	Visiting Fellow	LCB, NIDDK
	Alan N. Schechter	Chief	LCB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

0.2

## PROFESSIONAL:

0.2

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- |   |   |                                      |
|---|---|--------------------------------------|
| <input type="checkbox"/> (a) Human subjects | <input checked="" type="checkbox"/> (b) Human tissues | <input type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors        |   |                                      |
| <input type="checkbox"/> (a2) Interviews    |   |                                      |

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

This project has been combined with Z01 DK 25016-16 LCB.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 25056-04 LCB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (30 characters or less. Title must fit on one line between the borders.) <u>Regulation of Human T Cell Receptor Delta and Alpha Gene Usage</u>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Jean-Pierre de Villartay	Visiting Fellow                      LCB, NIDDK
Others:	David Cohen	Medical Officer                      LCB, NIDDK
COOPERATING UNITS (if any) Washington University School of Medicine (Dr. S. Korsmeyer); Lab. of Tumor Cell Biology, NCI (Dr. E. Tschachler) Metabolism Branch, NCI (Drs. C. Glenn Begley, M. P. Davey, T. A. Waldmann), Navy Medical Oncology (Dr. Ilan R. Kirsch)		
LAB/BRANCH Laboratory of Chemical Biology		
SECTION Section on Molecular Biology and Genetics		
INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland		
TOTAL MAN-YEARS	PROFESSIONAL	OTHER:
1.5	1.5	
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>The ability of T lymphocytes to recognize diverse ligands (antigens) resides in the T cell receptor (TCR), which is a heterodimer constructed from somatically rearranging variable (V) diversity (D) and joining (J) elements to account for its diversity, while each gene contains an invariant constant region. Most mature, effector T lymphocytes express the same class of TCR constant region, termed alpha/beta, but a smaller subclass of T cells appearing early in thymic ontogeny has been found to express a different heterodimer, termed gamma/delta. The function, the ligand, and the genetic regulation of this second receptor has been previously unknown although genetic clones for the gamma TCR existed. This project seeks to define and clone the delta TCR gene encoding the remaining chain of the second heterodimer, and to understand how genetic selection of the alpha/beta vs. gamma/delta TCR is accomplished.</p> <p>Our laboratory and two other laboratories independently cloned the delta TCR gene utilizing the discovery that the delta TCR gene is tightly linked to the alpha TCR gene. These studies have established that the genetic order on human chromosome 14 is V-D-J delta-C delta-J alpha-C alpha, meaning that components of the alpha TCR flank the delta TCR on both sides. We have discovered an important regulatory rearrangement which deletes the delta TCR and which appears to be the initial step in alpha TCR gene usage. Consequently, a cell selects either gamma/delta or alpha/beta gene usage, so that these T cells derive from two different cell lineages. The regulatory rearrangement shares many common features with two immunoglobulin regulatory recombination events; (class switching and kappa gene deletion). The discovery of this regulatory rearrangement in a very immature T cell tumor is consistent with the premise that it is an early event distinguishing two developing lineages of T cells.</p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 25057-04 LCB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Function, Ligand, and Ontogeny of Expression of the Gamma/Delta T Cell Receptor		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.:	Amy B. Pullman	Guest Researcher LCB, NIDDK
Others:	David I. Cohen	Medical Officer LCB, NIDDK
	J. P. de Villartay	Visiting Fellow LCB, NIDDK
	Lisa Jacobs	Biologist LCB, NIDDK
COOPERATING UNITS (if any)  NIAID, NIH (Drs. J. Coligan and E. Shevach); NCI, NIH (Dr. Jeffrey Cossman); FDA (Dr. L. Matis).		
LAB/BRANCH Laboratory of Chemical Biology		
SECTION Section on Molecular Biology and Genetics		
INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland		
TOTAL MAN-YEARS: 2.0	PROFESSIONAL: 1.5	OTHER: .5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>Effector T lymphocytes recognize antigens in association with a product of the major histocompatibility complex (MHC) through the T-cell receptor for antigen (TCR), a heterodimer composed of disulfide-linked alpha and beta chains. The appropriate pool of alpha/beta TCR-bearing cells is generated in the thymus upon positive and negative selection, a process which eliminates TCRs with a high affinity for self MHC alone while expanding T cells bearing TCRs with affinity for self in association with foreign antigens. Although some progress in understanding this process has occurred, the precise mechanism of thymic education remains poorly understood. In addition to the alpha/beta TCR, a second TCR, termed gamma/delta, has been described on the surface of a small subset of cells and thymocytes. The function and the ligand(s) of the gamma/delta TCR remains unclear, although cells using this receptor have been associated with MHC-restricted and non-restricted cytotoxicity. Because of its expression of immature T cells and thymocytes, it has been proposed that the gamma/delta TCR may play a role during the early events of thymic T cell development, including thymic education and T cell tolerance induction. This project aims to define the function, ligand(s), and ontogeny of expression of the gamma/delta TCR both murine and human models.</p> <p>In order to dissect this project at the molecular level, we have first sought to define the complexity of the variable (V), diversity (D), and joining (J) element usage for the newly-described human delta TCR gene, because these elements combine to form the ligand-binding part of the receptor. We analyzed the gene arrangements associated with the newly described <math>\delta</math> TCR gene from a series of 19 consecutive precursor T-cell (lymphoblastic) neoplasms that represent discrete stages surrounding the TCR gene rearrangement process. Significantly, the <math>\delta</math> TCR gene showed rearrangement in most (13/19) of these T-cells, and in addition, it was rearranged in two cells displaying no rearrangement for any other TCR gene. While only a single, productive V<math>\delta</math>-J<math>\delta</math>-C<math>\delta</math> joining has previously been identified in man, our survey revealed two additional types of <math>\delta</math> gene rearrangements associated with cell-surface TCR expression that presumably represent novel V gene usage. This analysis demonstrates 1) a major subclass of human precursor T-cell neoplasms belonging to the <math>\gamma\delta</math> T-cell receptor-rearranging subtype 2) a narrow repertoire of human V<math>\delta</math> gene usage and 3) the utility of <math>\delta</math> gene rearrangements as a diagnostic clonal marker in precursor T lymphoblastic neoplasms.</p> <p>There are now a total of six V genes, three D genes and one principal J gene known to contribute to the complexity of the human gene.</p> <p>A genomic clone was derived for one of the new V genes, while the other V gene rearrangement was not detected by the J<math>\delta</math>1 probe, consistent with the possibility that this cell uses a J<math>\delta</math> segment distinct from the one analyzed here, such as J<math>\delta</math>2.</p> <p>Genomic clones for the D &amp; J regions are being developed to study the ontogeny of the gene's expression in human thymus. In addition, a series of precursor B cell leukemias was analyzed for rearrangements at the <math>\delta</math> chain gene. Human precursor B cell acute-lymphoblastic leukemias (ALL) have previously been known to frequently rearrange their TCR <math>\alpha</math>, <math>\beta</math> and <math>\delta</math> chain genes. We found that the majority of precursor B cell ALL (12 of 16) showed rearrangement or deletion of one or more TCR <math>\delta</math> genes in contrast to mature B cell neoplasms in which no TCR <math>\delta</math> gene rearrangements were detected.</p>		

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25058-04 LCB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Laboratory and Clinical Models for the Study of Globin Gene Expression

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Griffin P. Rodgers	Senior Staff Fellow	LCB, NIDDK
Others:	Constance T. Noguchi	Research Physicist	LCB, NIDDK
	Moshe Mittelman	Visiting Scientist	LCB, NIDDK
	Alan N. Schechter	Chief	LCB, NIDDK

## COOPERATING UNITS (if any)

MRC Unit, Univ. of West Indies, Kingston, Jamaica (Dr. G. Serjeant).

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

0.3

## PROFESSIONAL:

0.3

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We are investigating the molecular mechanisms which control the individual and total concentrations of hemoglobins in human erythrocytes. In addition, we are studying the effects of functional alpha globin gene number, fetal hemoglobin (HbF) levels and the extent of red cell heterogeneity on the various manifestations of sickle cell disease and its genetic variants. The levels of each of the normal hemoglobins (A, A2, F) are determined by controls at the level of transcription and/or translation of the globin genes, as well as by factors that regulate protein degradation. The study of the control of hemoglobin levels has direct relevance to various hemoglobinopathies, especially thalassemia and sickle cell disease. For our experimental system, we are using the K562 human leukemic cell line, as well as peripheral blood from individuals with sickle cell disease. We are studying the effects of short-term and long-term exposure of these cells to 5-azacytidine and hemin on their phenotype and the factors that control globin gene transcription. Adult beta-mRNA expression remains undetectable, yet we have found a constitutive level of another adult type hemoglobin, delta-mRNA, whose expression is inducible both with hemin and 5-azacytidine. Because of the close sequence homologies between the delta- and beta-globin genes, experiments are underway to examine whether changes in the delta-promoter sequence may alter important protein binding sites and thereby result in the low levels of delta-globin gene expression. Identification of these putative protein(s) binding sites may not only provide important information on the regulation of the minor hemoglobin synthesis but may allow for the characterization of trans-acting factor(s) responsible for beta-globin gene expression.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Zol DK 25059-04 LCB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <u>Trans-activating Factors and Globin Gene Expression: A Direct Approach</u>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Harish Dave  Others: Shi-Xian Cao Pablo Cutman Alan N. Schechter	Visiting Fellow  Visiting Associate Visiting Fellow Chief  LCB, NIDDK  LCB, NIDDK LCB, NIDDK LCB, NIDDK
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Chemical Biology		
SECTION Section on Molecular Biology and Genetics		
INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland		
TOTAL MAN-YEARS: <div style="text-align: center;">0.5</div>	PROFESSIONAL: <div style="text-align: center;">0.5</div>	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>Humans undergo two developmental switches in their hemoglobin phenotype. The embryonic to fetal switch early in gestation and the fetal to adult switch around the time of birth. The K562 human leukemia cell line expresses all globin genes other than the adult beta-globin. Previous work from this laboratory has shown that the K562 beta-globin gene functions normally in a heterologous expression system. Elucidation of the mechanism of failure of beta-globin gene expression in K562 cells may provide an insight into globin gene expression and switching in normal erythroid cells.</p> <p>A stable transferrant assay has been established to study the localization of sequences conferring tissue specificity to the upstream region of globin genes. We have shown that the 104 base pairs 5' of the cap site of the epsilon-globin gene are sufficient for tissue specific expression.</p> <p>A new human cell line, SP1-802, has been characterized. This line displays a mixed embryonic/fetal phenotype and shows activation of the <math>\epsilon</math>, <math>\gamma</math>, <math>\zeta</math> and <math>\alpha</math> globin genes. There is a marked induction of <math>\gamma</math> globin gene expression upon exposure of the cells to hemin. This line represents a useful addition to the armamentarium of human erythroid cell lines and provides a laboratory model for testing of agents that result in induction of the <math>\gamma</math> globin gene.</p> <p>Stable transformants of K562 cells containing integrated constitutively expressing HTLV-1 tat genes have been developed. These cells display a stimulation of <math>\alpha</math>, <math>\zeta</math>, <math>\epsilon</math> and <math>\gamma</math> globin genes. This is correlated with increased % benzidine positivity and spectrophotometrically measured hemoglobin.</p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 25060-04 LCB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <u>In Vitro Transcription of Human Globin Genes With K562 Nuclear Extracts</u>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Yuko Wada	Visiting Associate LCB, NIDDK
Others:	Constance T. Noguchi	Research Physicist LCB, NIDDK
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Chemical Biology		
SECTION Section on Molecular Biology and Genetics		
INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)  <p>           In order to study the regulatory role of <u>trans</u>-acting factors and <u>cis</u>-acting DNA sequences in human globin gene expression, we have employed the <u>in vitro</u> transcription (run-off) assay based on nuclear extracts from the human erythroleukemic K562 cell line as a complete homologous system. <math>\epsilon</math>-globin and <math>\gamma</math>-globin genes which are expressed in K562 cells, were actively transcribed by K562 nuclear extracts <u>in vitro</u>. In contrast, <math>\beta</math>-globin which is not expressed in K562 cells, was not transcriptionally active in K562 nuclear extracts. However, when K562 nuclear extract was supplemented with extract from cytoplasm of K562 cells or with HeLa whole cell extract, transcripts produced from a <math>\beta</math>-globin DNA template were detected. The transcriptional activation of <math>\beta</math>-globin upon the addition of cytoplasmic extract suggests that factor(s) required for <math>\beta</math>-globin gene expression may be produced in the cytoplasm but is not transported to the K562 nucleus, thereby preventing <math>\beta</math>-globin gene transcription <u>in situ</u>.         </p> <p>           For the identification and isolation of <u>trans</u>-acting factors for <math>\epsilon</math>-globin gene expression, the K562 nuclear extract was further fractionated by ion exchange chromatography (DEAE Sepharose) by means of a step-wise elution with ammonium sulfate. Only the 175 mM fraction (F175) was able to direct accurate transcription from <math>\epsilon</math>- and <math>\gamma</math> globin gene promoters. Inhibition of transcription by <math>\alpha</math>-amanitin confirmed that the transcription by F175 is class II (driven by polymerase II).         </p> <p>           To know the importance of <u>cis</u>-acting promoter sequence in regulation of transcription of the <math>\epsilon</math>-globin gene, several proximal 5' upstream deleted DNA templates were employed for <u>in vitro</u> transcription assay. Preliminary results indicate that run-off transcripts from DNA templates which included silencer sequences were detected only at low levels with K562 unfractionated nuclear extract, but were clearly detectable with K562 F175 extract.         </p> <p>           Ribonuclease T1 analysis demonstrated that accurate initiation of transcription from a DNA template which has the ATA box but not the CAT box could not be observed. The hybridized signal from ribonuclease T1 analysis correctly initiated transcripts was lowest using DNA templates which included the <math>\epsilon</math>-globin silencer region. However, such a significant difference in expression among the deleted DNA templates could not be observed when F175 extract was employed. These studies suggest that protein factors which interact with the <math>\epsilon</math>-globin silencer region, including the inhibitory activity, can be fractionated by ion exchange chromatography.         </p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 25061-04 LCB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Isolation of Embryonic Globin Transcriptional Factors by Subtractive cDNA Cloning		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Yongji Wu	Visiting Associate                      LCB, NIDDK
Others:	Constance T. Noguchi	Research Physicist                      LCB, NIDDK
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Chemical Biology		
SECTION Section on Molecular Biology and Genetics		
INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>The specific transcription of human globin genes may involve a complex interaction of a variety of factors including trans-acting factors, none of which has thus far been completely characterized. The K562 human erythroleukemia cell line can serve as a model for the study of globin gene expression. The goal of the present study is trying to clone and characterize such factors.</p> <p>The current study assumes that induced K562 cells contain transcriptional factors specific for embryonic and fetal globin genes, which are absent or present only at very low levels in uninduced K562 cells. For isolation of cDNA clones encoding the trans-acting factors, a cDNA library from mRNA of induced K562 cells, consisting of 450,000 independent recombinants, was constructed and 150,000 recombinants has been differently screened. Seventy-five cDNA clones were found to hybridize only with cDNA probes from induced K562 cellular RNA and further examined by hybridization with Northern blot of induced and uninduced K562 cellular RNA. Forty-five cDNA clones have shown full length complements to the corresponding RNA. To determine whether some of the 45 cDNA encode trans-acting factors required to activate epsilon- or gamma-globin gene promoter, those cDNA have been inserted into Okayama-Berg expression vector and co-transfected into HeLa cells with another expression vector which contains a reporter gene (CAT or hGH) driven by E or R promoter. Six cDNA clones have been shown to be able to increase CAT and hGH gene expression 3 to 6 times. Sequences of 4 cDNA (No. #1, #8, #17, #35) of the 6 cDNA's show homology to human ferritin heavy chain cDNA sequence; 1 (No. #27) of the 6 cDNAs shows homology to human ferritin light chain cDNA sequence; 1 (No. #52) of the 6 cDNAs shows no homology to any known cDNA or DNA sequences in Genbank. These results suggest that ferritin not only plays an important role in cellular iron storage and utilization that have been well known, but also in regulation of globin gene expression through activating globin gene promoter. #52 cDNA may encode a protein unknown before and involved in the activation of the globin gene promoter. We are now working on the mechanism by which ferritin cDNA and #52 cDNA function.</p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 25063-03 LCB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders) Effect of Hydroxyurea on Fetal Hemoglobin Synthesis in Sickle Cell Patients		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Griffin P. Rodgers	Senior Staff Fellow LCB, NIDDK
Others:	Shu-zhen Huang Constance T. Noguchi Alan N. Schechter	Courtesy Associate LCB, NIDDK Research Physicist LCB, NIDDK Chief LCB, NIDDK
COOPERATING UNITS (if any) CHB, NHLBI (A.W. Niehnuis); CB, NEI (Dr. M. Roy); BEIB (Mr. E. Walker); Depts. of Medicine, Pediatrics & Pathology, Johns Hopkins University, Baltimore, Md. (Dr. G. Dover); Laboratory of Medical Genetics, Shanghai Children's Hospital, Shanghai China (Drs. Y. Zeng and S. Huang)		
LAB/BRANCH laboratory of Chemical Biology		
SECTION Section on Molecular Biology and Genetics		
INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.4	0.4	
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>We have focused on pharmacological augmentation of fetal hemoglobin levels in sickle cell patients. In particular, hydroxyurea (HU), a cell-cycle specific agent that blocks DNA synthesis by inhibiting ribonucleotide reductase, has been shown to increase fetal hemoglobin (HbF) levels in some patients with sickle cell disease, although the mechanism of action remains to be defined. In order to develop protocols for effective treatment of sickle cell patients, we have studied the effects of HU administration on ten hospitalized patients treated on an escalating dose schedule for periods of three months. Of the ten patients, 7 were considered responders by virtue of at least a two-fold increase in the %F-reticulocytes and a concomitant two-fold rise in %HbF. Among the responders, HbF levels increased 2 to 10-fold, generally after a lag period of about 40 days (range - 10 to 65 days). Three patients achieved levels of HbF of 10 to 15%. The initial values of HbF, F-retics, the degree of anemia or the specific beta-globin gene haplotype were not predictive of response. Statistical analysis of the three cellular variables that determine HbF levels in these patients disclosed that HbF production, as estimated by F-retic number, accounted for the major part of the increase in HbF. Four of the responders were retreated with optimal HU dose after a "washout" period and were found to have greater HbF responses, again occurring after a measurable lag period. This prolonged lag period prior to response suggests that mechanisms other than acute cyto reduction with regeneration, such as changes in mechanisms controlling gamma-globin gene transcription may be operative in the increased HbF synthesis.</p> <p>Should a significant sustained F-cell response in select patients while on HU, it may be possible to increase further the magnitude of the response by simultaneously administering short courses of cloned human erythropoietin or cloned granulocyte-macrophage colony stimulating factor. In this fashion, one may approach fetal hemoglobin levels consonant with those observed in the benign HbS-HPFH phenotypes.</p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 25064-03 LCB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Cytogenetic Investigations of Patients with Genetically Determined Disorders		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Beverly J. White	Director, Cytogenetic Unit LCB, NIDDK
Others:	Mary Graham	Medical Technologist OD, CC
	Christopher Reed	Chemist OD, CC
	Hugo Deinken	Medical Aid OD, CC
	Berj Kalayjian	Medical Aid OD, CC
COOPERATING UNITS (if any) Med. Genetics Program, CC (J. Mulvihill, D. Parry, J. Green); Genetics Dept., Children's Hospital Natl. med. Ctr., Washington, D.C. (K. Rosenbaum); LN, NIA (M. Schapiro, S. Rapoport); CNG, NIMH (L. Delisi, E. Gershon); DMNB, NINCDS (S. Carlissen, B. Dropulic).		
LAB/BRANCH Laboratory of Chemical Biology		
SECTION Molecular Forces and Assembly (Cytogenetics Unit)		
INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.7	0.3
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>In cooperation with the medical genetics program, cytogenetic studies of 394 clinical center patients were conducted and retrieved from a computerized database for correlation with clinical status. Karyotypes were abnormal in 20.8% of all patients. Cytogenetic diagnosis of 38 Turner syndrome patients was confirmed; chromosomal abnormalities were frequent in patients with short stature (19%), hypogonadism or infertility (14%), dysmorphic syndromes (14%), and premature ovarian failure (10.5%). Reported cases included: analysis of cultured tumor cells from a patient with familial parathyroid carcinoma, detection of mosaic translocation trisomy 21 in a non-mentally retarded woman with presenile dementia, and association of 47, XXY Klinefelter syndrome with a conduct disorder.</p> <p>A collaborative study of nucleolus organizing region (NOR) variants in Down syndrome parents was reported. Down Syndrome was not associated with specific types of variants, and we concluded that NOR analysis should not be used to detect individuals at high risk of having Down Syndrome offspring. A cytogenetic study of schizophrenic males was reported; none had abnormalities, and a previously reported association of a hereditary fragile site at Xq27 and symptoms of schizophrenia could not be confirmed.</p> <p>Five embryonic stem cell lines were karyotyped in preparation for experiments to develop a transgenic sickle cell mouse. One line had a sufficient number of cytogenetically normal cells for use in transgenesis; the remaining lines were aneuploid, with gains of different autosomes. Studies of growth factors which could be used to promote karyotypic stability of embryonic stem cell lines are planned.</p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE		PROJECT NUMBER
NOTICE OF INTRAMURAL RESEARCH PROJECT		Z01 DK 25066-03 LCB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) AIDS: Transcriptional Regulation by TAT-Protein and LTR of HIV In Vitro		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Jiangang Yuan	Visiting Fellow LCB, NIDDK
Others:	Constance Noguchi	Research Physicist LCB, NIDDK
	Alan Schechter	Chief LCB, NIDDK
COOPERATING UNITS (if any) Kabigen, Stockholm, Sweden (Professor M. Hartmanis)		
LAB/BRANCH Laboratory of Chemical Biology		
SECTION Section on Molecular Biology and Genetics		
INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>The HIV retrovirus is the etiologic agent for AIDS. This virus appears to have developed highly unusual mechanisms which increases its virulence. One of these mechanisms is the existence of a strong transactivating gene, TAT, which markedly enhances the expression of viral genes. Although the TAT gene was recognized early in the analysis of the HIV genome, its mechanism of action is still unclear. It is likely that analysis of TAT protein function will require development of more direct assays.</p> <p>We have previously shown by using <u>in vitro</u> transcriptional assays that the nuclear extract of HeLa/t2 cell, which constitutively expresses TAT protein, markedly enhanced transcription from the HIV-LTR promoter. In order to be able to provide more directly evidence for the transacting activity of the TAT protein, we have continued our <u>in vitro</u> transcription assay by using purified TAT protein from different sources. The results showed that when purified TAT protein was added into <u>in vitro</u> transcription reaction mixture, the specific RNA product from HIV-LTR was significantly increased. We also determined the relation between the activity of TAT protein and the TAR region (transactivating response sequence of HIV-LTR) by using competition assay and gel retardation assay. Our preliminary experiments suggest that the TAT protein does not directly interact with the TAR region. We are now using a modification of this assay to develop other assays to screen potential inhibitors of TAT activity. This work could lead to a new approach to the prevention or treatment of AIDS.</p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 25067-02 LCB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <u>Genetic Control and Mechanism of Action Erythropoietin</u>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI:                      Henry B. Fox                      Staff Fellow                      LCB, NIDDK		
COOPERATING UNITS (if any) AFRRI, Defense Nuclear Agency, Bethesda, Maryland (Dr. W.D. Hankins and Ms. K. Chin)		
LAB/BRANCH <u>Laboratory of Chemical Biology</u>		
SECTION Section on Molecular Biology and Genetics		
INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  This project has been terminated.		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 25068-02 LCB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Regulation of Globin Gene Expression by Upstream Positive Control DNA Sequences		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Moshe Mittelman	Visiting Associate LCB, NIDDK
Others:	Patricia Berg	Senior Staff Fellow LCB, NIDDK
	Alan N. Schechter	Chief LCB, NIDDK
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Chemical Biology		
SECTION Section on Molecular Biology and Genetics		
INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.4	1.4	
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>             We are focusing on the role of the distal promoter (up to -500 nucleotides) in the regulation of the human beta globin gene. Previous experiments (project #Z01 DK 25045-05 LCB), were based on transient expression assays, in which recombinant plasmids, containing a series of deletion mutants of the 5' flanking region of the beta globin, linked to the CAT reporter gene and to the termination signal of the SV40, were transfected into K562 erythroid cells, followed by CAT assays. These experiments suggested that the region from -233 to -185 (the -200 region) stimulates the beta globin expression. Of note is that this region contains a consensus sequence for binding of nuclear factor I (NF1), a protein which was found to be necessary for the replication of the DNA of adenovirus, and appears to be indistinguishable from the CAAT box transcription factors (CTF). Another, although partial and hence weaker NF1 site is located in the -150 region.           </p> <p>             The results, so far, can be summarized as follows: 1) There is a protein-DNA binding, and mapped to the NF1 site (by both gel shift competition assays and DNase I footprinting assay). 2) Purified NF1 resulted in a similar gel shift pattern to K562. 3) Antibody anti-NF1 confirmed the involvement of the NF1 in this binding. 4) Initial data suggest that NF1 interacts with other factors in potential regulation of the human <math>\beta</math>-globin gene.           </p> <p>             In the current project, we are focusing on a detailed structural analysis of the above region. We are looking for putative <u>cis</u>-regulatory sequences, recognized by potential regulatory proteins (<u>trans</u>-acting factors), as well as trying to identify these factors.           </p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 25069-01 LCB												
PERIOD COVERED October 1, 1988 to September 30, 1989														
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Identification of Regulatory Elements Within the Human Alpha-2 Globin Promoter														
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI:</td> <td style="width: 33%;">Frank Shafer</td> <td style="width: 33%;">Guest Worker</td> <td style="width: 33%;">LCB, NIDDK</td> </tr> <tr> <td>Others:</td> <td>Constance T. Noguchi</td> <td>Research Physicist</td> <td>LCB, NIDDK</td> </tr> <tr> <td></td> <td>Alan N. Schechter</td> <td>Chief</td> <td>LCB, NIDDK</td> </tr> </table>			PI:	Frank Shafer	Guest Worker	LCB, NIDDK	Others:	Constance T. Noguchi	Research Physicist	LCB, NIDDK		Alan N. Schechter	Chief	LCB, NIDDK
PI:	Frank Shafer	Guest Worker	LCB, NIDDK											
Others:	Constance T. Noguchi	Research Physicist	LCB, NIDDK											
	Alan N. Schechter	Chief	LCB, NIDDK											
COOPERATING UNITS (if any)														
LAB/BRANCH Laboratory of Chemical Biology														
SECTION Section on Molecular Biology and Genetics														
INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland														
TOTAL MAN-YEARS: <div style="text-align: center;">1.1</div>	PROFESSIONAL: <div style="text-align: center;">1.1</div>	OTHER:												
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div> <input type="checkbox"/> (a) Human subjects  <input type="checkbox"/> (a1) Minors  <input type="checkbox"/> (a2) Interviews         </div> <div> <input type="checkbox"/> (b) Human tissues         </div> <div> <input checked="" type="checkbox"/> (c) Neither         </div> </div>														
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Regulation of transcription of the human globin genes is both tissue and developmental specific. Expression of alpha-like and beta-like globin genes begin during the third week of embryogenesis and are produced in the yolk sac through eighth weeks of gestation. From the eighth to the twenty-eighth week, the liver becomes the major organ of erythropoiesis and globin expression shifts toward the fetal and adult globins. By birth, the red cell production has shifted from the liver and spleen to the bone marrow. Human globin expression shifts to almost entirely adult globins by six months post natal age. The complex interplay of cis acting DNA globin sequences and trans-acting protein factors and their role in regulating gene expression is not well understood. A detailed explanation at the molecular level will provide insight into the temporal and spatial specificity of gene expression. To this end we are studying the regulation of expression of the alpha globin gene by determining structural and functional properties of the alpha globin gene cluster. The K562 human erythroleukemia cell line will serve as a model for the study of gene expression since this cell line contains transcriptional factors necessary for the developmental expression of the human alpha globin gene. The current study focuses on defining regions within the 5' promoter region of the alpha-2 globin gene that act as enhancer or repressor elements regulating gene expression. Initially, the region being examined includes 575 base pairs upstream of the alpha globin reading frame extending to the PST I restriction enzyme site. Serial deletion mutations of the 5' alpha globin promoter region will be carried out and cloned into an expression vector using either the interleukin-2 receptor (IL2R) or firefly luciferase as reporter genes. Using a monoclonal antibody to IL2R, the amount of IL2R expressed on the cell surface will be assayed using fluorimetry. This assay technique would avoid the necessity to lyse cells. When the luciferase gene is used as the reporter, the amount of luciferase produced will be monitored by luminescence. In later studies, this analysis will be extended to include the extreme 5' regions of the alpha globin cluster.</p>														

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 25070-01 LCB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Analysis of the Epsilon Globin Gene Flanking Sequences		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI:                      Natalya Merezhinskaya                      Visiting Fellow                      LCB, NIDDK		
Others:                      Constance T. Noguchi                      Research Physicist                      LCB, NIDDK Alan N. Schechter                      Chief                      LCB, NIDDK		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Chemical Biology		
SECTION Section on Molecular Biology and Genetics		
INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland		
TOTAL MAN-YEARS  1.0	PROFESSIONAL  1.0	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>K 562 is an erythroleukemic cell line which expresses embryonic epsilon- and fetal gamma- but not adult beta- globin genes. Regulation of the globin gene family includes cis- acting DNA sequences which are thought to control tissue- and development- specific expression of these genes. Our goals are to define functionally active DNA sequences in 5' flanking region of epsilon- globin gene, to show the specific interaction of regulatory protein(s) with these regions, and to isolate and to characterize these specific DNA-binding protein(s).</p> <p>The first step of our study will include the determination of functionally important 5' flanking DNA sequences by deletion analysis of 2 Kb upstream region of the epsilon- globin gene. These 5' flanking DNA sequences will be fused to reporter gene corresponding to a protein which can be readily quantitated in the cytoplasm, can be excreted into the media or can be expressed on the surface of the cell. The level of reporter protein will be an indication of the transcription activity of the epsilon-globin 5' flanking sequence. The functional activity of these DNA sequences will further be proven by methylation and direct mutations. Experiments leading to the detection of protein interaction site(s) will include DNase footprinting and gel retardation assays. Finally, attempts will be undertaken to isolate and characterize regulatory protein(s) which specifically interact with DNA sequences of the 5' flanking epsilon-globin region.</p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 25071-01 LCB
PERIOD COVERED October 1, 1988 to September 1, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Trans-regulation of Human Globin Genes		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Robert H. Broyles	Guest Worker  LCB, NIDDK
Others:	Patricia Berg Alan N. Schechter	Senior Staff Fellow Chief  LCB, NIDDK LCB, NIDDK
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Chemical Biology		
SECTION Section on Molecular Biology and Genetics		
INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither		
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>             An understanding of the molecular mechanisms that regulate developmental hemoglobin (hb) switching would be useful to the goal of increasing fetal Hb in adult humans with sickle cell disease, a manipulation that is likely to alleviate most if not all clinical manifestations of the disease. As part of a project which has as its goal the identification and isolation of <u>trans</u>-acting factors that regulate developmental Hb switching, we are seeking to identify and isolate protein regulatory factors that bind to the 5' noncoding regions of the human beta globin gene.           </p> <p>             The following observations seem important to us and have led to the hypothesis stated below: (1) K562 cells have a number of characteristics of authentic embryonic erythroid cells including their ability to express embryonic and fetal globin chains and their substantial amount of ferritin. (2) Although K562 cells have an unmutated beta globin gene that is sensitive to DNase I, they do not express this adult globin gene. (3) The upstream (5' noncoding) region of the human beta globin gene contains a DNA version of the consensus sequence of the <u>iron responsive element</u> (IRE) involved in the translational control of ferritin mRNA. The hypothesis proposed here is that (a) ferritin (in one or more of its forms or subunits) is a negative regulator of the beta globin gene and (b) ferritin mediates its effect by binding to this recently observed IRE consensus sequence. Part (b) is presently being tested.           </p> <p>             Binding of ferritin to the Rsa I fragment of the beta globin 5' noncoding region which contains the IRE sequence is being assayed with gel retardation assays. Preliminary results show that H and L ferritin bind to the Rsa fragment whereas transferrin and apotransferrin do not. Further experiments to test the optimal conditions and specificity of this binding are underway. Gel shift competition experiments and DNA footprinting will be used to determine the sequence to which the ferritin is binding.           </p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 25072-01 LCB
PERIOD COVERED October 1, 1988 to September 30, 1989.		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Control of Transcription of Erythropoietin		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	W. David Hankins	Guest Worker LCB, NIDDK
Others:	F. Xu	Guest Worker LCB, NIDDK
	Kyung Bae	Guest Worker LCB, NIDDK
	Alan N. Schechter	Chief LCB, NIDDK
COOPERATING UNITS (if any) Kidney Section, NIDDK (Drs. Gary and Lillian Striker); AFRRRI, Bethesda, Maryland (Dr. T. MacVittie)		
LAB/BRANCH Laboratory of Chemical Biology		
SECTION Section on Molecular Biology and Genetics		
INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.2	2.2	
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>The kidney is generally recognized as the major production site of erythropoietin (Epo), the physiologic regulator of red cell production. Experimental hypoxia produces a sharp increase in kidney Epo mRNA levels and serum Epo. The cellular oxygen sensing mechanism, the Epo specific transcriptional activating factors, and the genomic sequences necessary for Epo regulation have not been elucidated. In 1986, we isolated two cell lines which inappropriately synthesized Epo. To explain the abnormal Epo transcription and gain new information concerning Epo regulation, we will investigate the following three hypotheses:</p> <p>1). Genomic alterations removed negative regulatory sequences or inserted positive regulatory sequences; 2) Epo mRNA stability has increased, and 3) putative Epo transcriptional activating factors have been inappropriately expressed.</p> <p>We have explored the first model by restriction mapping and have discovered genomic alterations in the Epo genes from both cell lines. In one case, the alterations are upstream of Epo coding sequences. Current cloning and sequencing experiments are expected to reveal the precise nature of the Epo rearrangements. In a second project we are attempting to identify normal cells which adjust Epo transcription in response to changes in oxygen concentration <u>in vitro</u>. We will assess Epo inducibility in several new human kidney cell lines recently isolated at NIH.</p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 25073-01 LCB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) The erythropoietin Receptor and its Genetic Control Red Cell Development		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	W. David Hankins	Guest Worker LCB, NIDDK
Others:	Kyung Bae	Guest Worker LCB, NIDDK
	F. Xu	Guest Worker LCB, NIDDK
	Constance T. Noguchi	Research Physicist LCB, NIDDK
	Alan N. Schechter	Chief LCB, NIDDK
COOPERATING UNITS (if any) MIT (Dr. Alan D'Andrea); AFRRRI (Dr. Tom MacVittie); NIC (Dr. Sandra Ruscetti)		
LAB/BRANCH Laboratory of Chemical Biology		
SECTION Section on Molecular Biology and Genetics		
INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>The kidney hormone erythropoietin (Epo) specifically interacts with the red cell lineage and has been shown to influence DNA, RNA and hemoglobin synthesis in erythroid precursors. Therefore, it is likely that the expression of an Epo receptor plays a pivotal role during red cell development. Two recent events have significantly increased our ability to approach the structure, function, and regulation of this receptor. One was our isolation of unique cell lines which are exclusively dependent on Epo for survival, proliferation and hemoglobin synthesis. Second, the Epo receptor was molecularly cloned. In an attempt to gain information about the Epo receptor, we have initiated a multi-faceted program which will begin to address the following questions: 1) Is receptor expression, transient, inducible or constitutive during erythroblast development? 2) Does the receptor convey new instructions to a cell, provide a trigger for preprogrammed events, induce specific genes or process or serve as a permissive, viability factor for the cells, and 3) how is receptor expression regulated?</p> <p>The initial studies with HCD cells have demonstrated low affinity binding sites, Epo receptor mRNA and Epo internalization and metabolism. Certain retroviruses have been shown to abrogate the hormone-dependence in HCD cells but not in IL-3 dependent cells. Planned studies include determination of transcriptional regulator sequences, retroviral receptor transfer, and temporal definition of receptor expression.</p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 25074-01 LCB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Mechanism(s) of Enhanced Gamma Globin Gene Expression in Patients		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Myung Nam	NRSA LCB, NIDDK
Others:	Griffin P. Rodgers	Senior Staff Fellow LCB, NIDDK
	Pat Berg	Senior Staff Fellow LCB, NIDDK
	Alan N. Schechter	Chief LCB, NIDDK
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Chemical Biology		
SECTION Section on Molecular Biology and Genetics		
INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland		
TOTAL MAN-YEARS	PROFESSIONAL	OTHER:
0.8	0.8	
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>           Several lines of clinical and experimental evidence suggest that elevated levels of fetal hemoglobin (HbF) may improve the clinical course of individuals with sickle cell disease and beta thalassemia. A number of cytotoxic drugs have been shown to enhance gamma globin synthesis (and HbF levels) in experimental animals and patients with hemoglobinopathies, although the mechanism of action of these agents have yet to be clearly delineated. The K562 human erythroleukemia cell line has been shown to express constitutively low levels of embryonic and fetal (but not adult hemoglobin, and can be reversibly induced to preferentially increase gamma globin gene expression in response to hydroxyurea. We are therefore using the K562 cell as a model system to understand the mechanism by which hydroxyurea (and similar agents) induces fetal hemoglobin synthesis. K562 cells have been grown in the presence of 25mM hemin and 0, 25, 50, 100 and 150 mM hydroxyurea. Nuclear extracts have been prepared from these and other non-erythroid (control) cells. A 326 bp fragment 5' of the normal <math>\gamma</math> gene has been subcloned into an expression vector, and will be used to examine the differential binding characteristics of the nuclear protein extracts. We will employ gel-retardation electrophoresis, DNA-footprinting (DNase I and methylation protection), and ion exchange and affinity chromatography to investigate the interaction of both specific and non-specific protein/<math>\gamma</math> gene promoter) DNA. It is hoped that the identification, characterization and purification of these putative binding proteins would not only extend the current knowledge of the molecular basis of the fetal to adult "switch", but also suggest a novel pharmacological approach to the reversal of this switch in several clinically significant hemoglobinopathies.         </p>		

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 25075-01LCB

## PERIOD COVERED

October 1, 1989 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Production and Characterization of HIV TAT

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Tiee-Cherng Shieh

Visiting Fellow

LCB, NIDDK

Others: Jian-gang Yuan

Visiting Fellow

LCB, NIDDK

Constance T. Noguchi

Research Physicist

LCB, NIDDK

Alan N. Schechter

Chief

LCB, NIDDK

## COOPERATING UNITS (if any)

Kabigen AB, Stockholm, Sweden (Dr. M. Hartmanis), LCP,  
NIDDK, (Dr. A. Gronenborn), University of Padova, Italy (Dr. C. DiBello)

## LAB/BRANCH

Laboratory of Chemical Biology

## SECTION

Section on Molecular Biology and Genetics

## INSTITUTE AND LOCATION

NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

1.2

## PROFESSIONAL

1.2

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

Human immunodeficiency virus type 1 (HIV-1), the cause of AIDS, contains in its genome sequences for the major retroviral proteins, gag, pol and env. HIV-1 also encodes five or more novel proteins that appear to act as regulatory factors. One of these, TAT, which is a powerful trans-activator of HIV gene expression, is essential for virus replication. TAT is 86 amino acids long, contains a cysteine-rich region followed by a highly basic region. During the past year, we have endeavored to produce large amounts of TAT protein for detailed structural and functional studies. To this end, we have been working to express TAT protein in *E. coli* cells by using a unique *E. coli* expression system in which the tat-protein coding region was fused to the coding region for the IgG binding domain of protein A (designated Z). The production of Z-Z-TAT protein from 50 liter fermenters has been facilitated by a collaboration with KaBiGen AB. The Z-Z-TAT fusion protein has been partially purified by use of IgG affinity chromatography and the Z-Z peptide was cleaved by hydroxylamine. The expected yield of purified TAT is about 50 mg. To further purify the TAT protein from what appears to be degradation products, we are using HPLC and gel filtration. As specific *in vitro* assays for TAT are not yet available, we began to produce large amounts of polyclonal antibodies to follow TAT purification, for use in Western blotting and to probe TAT function. For this purpose, we have produced synthetic peptides from the carboxyl- and amino-terminal and central portions of the HIV-TAT protein sequence. Antibodies to these peptides and to recombinant HIV-TAT have been produced in sheep and will be used to assist in the isolation and affinity purification of HIV-TAT as well as for biochemical and functional characterization.

ANNUAL REPORT OF THE LABORATORY OF CHEMICAL PHYSICS  
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The research in the Laboratory of Chemical Physics is primarily concerned with the investigation of problems in molecular and cellular biophysics. A variety of spectroscopic techniques are employed in these investigations, including nuclear and electron magnetic resonance, Raman and infrared spectroscopies, electric-field-induced linear dichroism, ultraviolet and visible microspectrophotometry, and time-resolved optical spectroscopy using nanosecond lasers. There is also a major effort in theoretical studies to complement the experimental work, including both analytic methods and the use of high speed computers in large scale calculations. The systems under study include nucleic acids, proteins, intact and model membranes, retinal photoreceptors, and various small prototypical biological molecules. Current research focusses on: the development of new methods in nmr; the structure of macromolecules in solution by two-dimensional nmr; the structure and dynamical behavior of nucleic acids and nucleoproteins; conformational, dynamical, and functional characteristics of model membrane systems; the dynamics of ligand binding and conformational changes in proteins; theoretical analysis of kinetics and dynamics in macromolecules; computer simulations of atomic motions in proteins; rheological properties of cell membranes; the molecular mechanism of excitation in photoreceptor cells and ionic processes in cell membranes; the gelation of hemoglobin S and its relation to the pathophysiology of sickle cell disease; the analysis of excited electronic states of polyenes in the vapor phase and in molecular beams; and the asymmetric synthesis and structure of metabolites; The following gives a brief summary of the major findings over the past year.

Bax and colleagues have made a major advance in the determination of the three-dimensional structure of proteins in solution. Marion, Kay and Bax have developed a heteronuclear three-dimensional pulse scheme that permits separation of the standard two-dimensional NMR spectra into a third dimension, the chemical shift of the heteronucleus (N15 or C13) directly attached to the hydrogen. With this method it will be possible to extend the molecular weight accessible to complete, three-dimensional structure determination by NMR to 20 kD, as evidenced by the results obtained so far for staphylococcal nuclease (18 kD), calmodulin (16.7 kD), and interleukin-1-beta (17kD).

Bax and colleagues have developed a number of additional methods for studying the structure and dynamics of molecules in solution. Bax, Kay, Torchia (NIDR), and Sparks have used multiple quantum techniques to measure more sensitive J correlation spectra as well as backbone J couplings that normally do not show resolvable J splittings. Kay, Bax, Sparks, and Torchia (NIDR) have compared the backbone conformation of the solution structure from

J couplings with those of the X-ray crystal structure for staphylococcal nuclease and found good agreement with the exception of 4 residues involved in intermolecular contacts in the crystal. Kay, Torchia (NIDR), and Bax have developed novel 2D NMR techniques to study the backbone dynamics of staphylococcal nuclease. They find no correlation between the order parameter and the type of secondary structure, although significant differences in the order parameter are measured along the polypeptide backbone. Large amplitude motions on a relatively slow time scale are observed in the loop region of the protein, where the active site is located.

Clore, Gronenborn, and colleagues have used NMR to solve the complete three-dimensional structure of two proteins - the wild-type and an active site mutant of hirudin and the C-terminal domain of cellulase. Work is in progress on a number of other proteins, including the DNA binding protein *ner* from phage Mu, human interleukin-1-beta (with Driscoll), human interleukin-8, human thioredoxin (with Forman-Kay), and a trypsin inhibitor from *Ascaris*. Complete backbone assignments have been made for all of these proteins and elements of secondary structure have been identified. Interleukin-8 has been shown to be a dimer in solution and intersubunit contacts have been identified. In these studies extensive use is being made of 3D heteronuclear NMR to resolve overlapping resonances.

Clore and Gronenborn have also made a number of improvements in the methodology of determining protein structures in solution by NMR. These include an analysis of the accuracy with which interproton distances can be determined, the demonstration that the interproton distance constraints are much more important than the empirical energy function of the molecular dynamics calculations in determining the convergence of the solution structures for nucleic acids, the development of a new method of simulated annealing which starts with a random array of atoms and overcomes the folding problem associated with real space methods, and the development of a new method of obtaining stereospecific assignments employing a data base search in conjunction with experimental NOE and coupling constant data that gives much better agreement among the resulting structures.

Becker and Khetrpal have used NMR methods to study molecules oriented in liquid crystals, and have demonstrated that the intensities of the spinning side bands can be used to determine the signs of the order parameters of the molecules, a fundamental quantity that is difficult to obtain by other methods. Becker has also carried out a new analysis of the fundamental vibrations of p-benzoquinone based on recent Raman and earlier infrared data that resolve previous ambiguities in the assignment of the spectrum.

Charney and colleagues are using electric-field-induced linear birefringence and linear dichroism to study the structure and dynamical properties of DNA and other biopolymers in solution. They find that the orientation decay of pulsed electric field dichroism of the A form of DNA is relatively insensitive to the environmental

ionic strength over the same range in which the dichroism decay of the B form is highly sensitive, indicating a difference in the flexibility of the two DNA conformations.

Levin, Lewis, and colleagues are using vibrational Raman and infrared spectroscopies to investigate the dynamical, conformational, functional, and thermodynamic properties of both model and intact membrane assemblies. Infrared studies on the effect of ethanol on liver plasma membranes of rats show that the adaptive response to alcohol intake results in increased membrane order, and that additional in vitro treatment induces significant increases in bilayer order. These effects are consistent with a model of partial chain interdigitation, or chain overlap, of the opposing membrane monolayers of the bilayer. Raman spectroscopy has been used to show that in multilamellar lipid dispersions containing a binary mixture three classes of lipids are distinguishable - pure domains of each component and boundary lipids between the bulk domains. Levin and Lewis have continued the development of a Fourier-transform Raman spectrometer which couples infrared Nd:YAG laser excitation to a Michelson interferometer. This approach enables the acquisition of Raman spectra on biological samples that were previously unobtainable because of fluorescence or decomposition effects.

Hofrichter, Henry, and Eaton have used time-resolved optical spectroscopy with nanosecond lasers to investigate the kinetics of ligand rebinding and conformational changes in hemoglobin. Studies on trout hemoglobin have characterized the kinetics of the transition from the R to T quaternary structure, the tertiary conformational changes within the R structure, and the geminate ligand rebinding. The results indicate that the difference in the tertiary conformation of the two quaternary structures decreases with increasing temperature, which is consistent with the decrease in the free energy difference between the two quaternary states. In a related project the oxygen binding curves of single crystals of hemoglobin in the T quaternary structure have been measured using a microspectrophotometer. These studies by Mozzarelli, Henry, and Eaton indicate that there is little or no cooperativity in the binding, confirming the most important prediction of the two-state allosteric model. As part of an ongoing investigation on sickle cell disease, Eaton and Hofrichter have completed a comprehensive account of the physics and physical chemistry of hemoglobin S gelation.

Hofrichter, Lozier, and Henry have studied the spectra of the photoproducts of both light- and dark-adapted bacteriorhodopsin using the transient spectrometer. Previous studies have treated the photocycle of this photoactive proton pump as unidirectional. By measuring very precise time-resolved spectra they have shown that there are significant back reactions at multiple points in the cycle.

Szabo has carried out a several theoretical investigations on the functional significance of dynamical processes. He has

performed a theoretical analysis of both transient and steady-state fluorescence in solution and membranes, from which he has clarified the relation among a variety of theories based on the diffusive model of the microscopic dynamics, and has shown that a unified description can only be given when the nonradiative lifetime of a quencher-fluorophore pair explicitly depends on the interparticle distance. By generalizing the virial expansion for equilibrium (static) properties to the non-equilibrium or time domain, Szabo, Zwanzig, and Agmon (DCRT) have extended the theory of fluorescence quenching into the high concentration regime. Szabo, Pastor (FDA), and Brooks (DCRT) have investigated the accuracy of various Langevin and molecular dynamics algorithms, from which they have developed specific strategies for optimally calculating temperature, pressure, and energy from trajectories.

Zwanzig has carried out theoretical studies on the dynamical properties of macromolecules. Zwanzig and Pastor (FDA) have developed an anisotropic bead model for the hydrodynamical interaction of a protein with its surrounding medium. This model should lead to a better understanding of protein dynamics and to more efficient computer simulations. Zwanzig has also begun a study of diffusion in complex systems, which involve multidimensional potential energy surfaces, bottlenecks or entropic barriers, or anisotropic diffusion. These are difficulties that arise in modelling a number of biophysical processes.

Hagins and Yoshikami have investigated the mechanism of visual transduction using a combination of microcalorimetric, electrophysiological, and optical imaging techniques. The effects of calcium deprivation on the q1 heat transient that occurs during light-induced shutdown of the sensory dark current has been studied in frog retinas. The results provide further evidence that the currently favored hypothesis that light regulates the dark current by reducing the cytoplasmic concentration of cGMP is not correct in its current formulation. Hagins, Ross (LMB, NIDDK), and Yoshikami have designed and partially built two new pyroelectric calorimeters that will extend the studies of heat transients by improving the time resolution and provide the capability to rapidly alter the composition of the medium. Yoshikami and Spring (NHLBI) have studied the diffusion of fluorescent dyes in the outer segments of frog retinas by image-intensified videomicroscopy, and have shown that the discs obstruct more than 95% of the outer segment cross-sections while permitting free lateral diffusion in the planes of the discs.

Kon has employed electron paramagnetic resonance (EPR) techniques to study the rheological properties of red cells. Kon and Fukushima have used the flow-spin label EPR method to investigate the mechanism of the loss of red cell deformability due to Heinz body formation. They have shown that it is not necessary for the entire inner surface of the cell membrane to be coated with Heinz bodies (aggregates of denatured hemoglobin) for a decrease in deformability to occur, as has been generally thought, but that the deformability decrease starts prior to the visual appearance

of Heinz bodies. They have also shown that with phenylhydrazine, the decreased deformability arises from binding of the Heinz bodies to the band III protein of the membrane cytoskeleton, while with monomethyl hydrazine the decreased deformability may result from the viscosity increase of the cell contents due to oligomerization of the hemoglobin.

McDiarmid has been carrying out experimental studies on the electronic structure and conformation of the excited states of small polyatomic molecules that are models for more complex biological molecules. These studies employ a variety of spectroscopic methods, including resonant visible-UV multiphoton ionization and photoacoustic spectroscopies. Three Rydberg series leading to the first ionization potential of cyclopentadiene have been identified, as well as the long-sought  $2A_1$  valence state. By comparing room temperature and low temperature spectra McDiarmid and Doering (Johns Hopkins U.) have assigned a controversial band of carbon disulfide as the singlet-triplet transition of the adjacent Rydberg band.

Ziffer and coworkers have carried out investigations on the preparation of chiral compounds and the assignment of their absolute stereochemistry. They have been able to determine the configuration resulting from double bond migration in derivatives of (E)-cyclooctene. A new synthesis of 1- and 2-hydroxy-3-methylcholanthrene was developed and used to prepare deuterated samples of these alcohols. Structures and stereochemistries were determined for photochemical and thermal rearrangement products of oxetanes obtained from the photochemical  $2 + 2$  cycloaddition of methyl benzoate and other simple arenecarboxylic acid esters to furan and certain 1,3-dienes.

Scientist Emeritus Weiss and Cook (U. Wisconsin) have carried out studies on the synthesis of polyquinanes that utilize the "Weiss" reaction. They have introduced substituents into predetermined sites of the primary products of the Weiss reaction as groundwork for the synthesis of additional polyquinanes. Weiss and collaborators have also determined the structure of the mold pigment, elsinochrome A, by X-ray crystallography, which confirm the structure previously proposed by Weiss on the basis of chemical and spectroscopic studies.

## DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

PROJECT NUMBER

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK-29001-17-LCP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular dynamics and vibrational characteristics of membrane assemblies

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Ira W. Levin Research Chemist LCP-NIDDK

Others:	E. Neil Lewis	Visiting Associate	LCP-NIDDK
	Mark Devlin	IRTA	LCP-NIDDK
	Burton J. Litman	Special Volunteer	LCP-NIDDK
	James L. Slater	IRTA	LCP-NIDDK

## COOPERATING UNITS (if any)

R. Adams, LCP-NIDDK; Clifford J. Steer, Medical School, Univ. of Minn.; C. Huang, School of Medicine, Univ. of VA; James S. Vincent, Univ. of MD.; Sherwin Straus, FDA; V. F. Kalasinsky, Mississippi State Univ.

## LAB/BRANCH

Laboratory of Chemical Physics

## SECTION

Section on Molecular Biophysics

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4.5

## PROFESSIONAL:

4.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Vibrational Raman and infrared spectroscopy are used to probe the dynamical, conformational, functional and thermodynamic properties of both model and intact membrane assemblies. Emphasis is placed on elucidating both lipid-lipid and lipid-protein interactions within the bilayer aggregate. For example, infrared spectroscopic techniques were used to characterize the in vivo and in vitro effects of ethanol on liver plasma membranes derived from alcohol treated rats. Spectral frequency shifts of the bilayer lipid chain methylene carbon-hydrogen symmetric stretching modes indicate that the adaptive response of the liver plasma membranes of alcohol treated animals results in increased membrane order. Additional in vitro ethanol treatment induces significant increases in bilayer order. These membrane effects are consistent with a bilayer model of partial chain interdigitation, or chain overlap, of the opposing membrane monolayers. Since these membranes do not exhibit the phenomenon of "tolerance", we suggest that the strict definition of this membrane effect be reevaluated.

The use of deuterium isotopically substituted hydrogen chains in multilamellar lipid dispersions provides a means for studying domain formation in bilayer membranes. For binary mixtures of dipalmitoyl-phosphatidylcholine- $d_{62}$  (DPPC) and dihexadecylphosphatidylcholine (DHPC), three classes of lipids are spectroscopically distinguishable; namely, pure DHPC domains, pure DPPC- $d_{62}$  domains and boundary lipids between the bulk domains.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK-29002-16-LCP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chemistry of natural compounds, and synthetic organic chemistry

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. : Ulrich Weiss

Research Chemist  
(Scientist Emeritus)

LCP-NIDDK

COOPERATING UNITS (if any) Prof. James M. Cook, Department of Chemistry, University of Wisconsin-Milwaukee; Prof. Lucio Merlini, Dipartimento di Scienze Molecolari Agroalimentari, Università degli Studi di Milano, Milan, Italy; Prof. Gianluca Nasini, Department of Chemistry, Politecnico di Milano, Milan, Italy.

## LAB/BRANCH

Laboratory of Chemical Physics

## SECTION

Office of the Chief

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1

## PROFESSIONAL:

1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In continued cooperation with Prof. J. M. Cook and his coworkers at the University of Wisconsin-Milwaukee, the synthesis of di- and polycyclic ring systems composed of fused cyclopentane rings ("polyquinanes") has been developed further. The approach chosen is based on the ready stereospecific formation of derivatives of cis-bicyclo[3.3.0]octane-3,7-dione (1) from 1,2-dicarbonyl compounds and esters of 3-oxoglutaric acid (the "Weiss reaction").

This year's effort was directed mainly towards the introduction of the maximum number of double bonds into the polyquinanes so obtained; the resulting products would be of great theoretical interest. However, the experimental difficulties are great, and the goal has not yet been reached. The possibility of introducing substituents into positions 2+4 of the bicyclo[3.3.0]octanedione system, suggested but not proven in the literature, has been established.

In cooperation with Profs. Merlini and Nasini, the structure of the mold pigment elsinochrome A, derived by us earlier, has been confirmed unequivocally by x-ray crystallography.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK-29005-15-LCP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Asymmetric synthesis: structure, stereochemistry, and NMR

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. : Herman Ziffer Research Chemist LCP-NIDDK

Others: Yulin Hu Visiting Fellow LCP-NIDDK  
Ayala Balan Visiting Scientist LCP-NIDDK

## COOPERATING UNITS (if any)

Drs. J. V. Silverton, NHLBI, Thomas S. Cantrell, American University

## LAB/BRANCH

Laboratory of Chemical Physics

## SECTION

Section on Molecular Biophysics

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.75

## PROFESSIONAL:

1.75

## OTHER:

0.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Derivatives of (E)-cyclooctene constitute an unusual series of molecules which maintain chiral ring structures in the absence of chiral centers. The unusual reactivities of these molecules were used to answer an interesting question, namely, what happens to the configuration of the ring in a reaction resulting in double bond migration? We found that the configurations about both the carbinol and the double bond were inverted during the reaction of one diastereomer of optically active (E)-1-hydroxy-2-cyclooctene under Mitsunobu reaction conditions (diethyl azodicarboxylate and p-bromobenzoic acid). The absolute stereochemistries of the starting alcohol and the p-bromobenzoate formed in the reaction were determined.

A new synthesis of 1- and 2-hydroxy-3-methylcholanthrene was developed by hydroboration of 3-methylcholanthrylene, which is readily available from 3-methylcholanthrene. This procedure was employed to prepare labeled (deuterated) samples of these alcohols.

Methyl benzoate and other simple arenecarboxylic acid esters have been found to undergo 2 + 2 photochemical cycloaddition at the carbonyl group to furans and certain 1,3-dienes. These additions afforded mixtures of oxetanes and their 2 + 2 cycloreversion products. The latter compounds were isolated and their structures determined.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01-DK-29006-19-LCP
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) The structure and dynamics properties of macromolecules		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I. : Elliot Charney	Research Chemist	LCP-NIDDK
Others: Martin Riehm	IRTA Fellow	LCP-NIDDK
COOPERATING UNITS (if any) H-H Chen, George Mason University, Fairfax, VA; Rodney Harrington, University of Nevada, Reno, NV; D. C. Rau, LCB-NIDDK; Sybren Wijmenga, University of Nijmegen, The Netherlands		
LAB/BRANCH Laboratory of Chemical Physics		
SECTION Section on Spectroscopy and Structure		
INSTITUTE AND LOCATION NIH, NIDDK, Bethesda, MD 20892		
TOTAL MAN-YEARS: 2.5	PROFESSIONAL: 2.0	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>Macromolecular structure, dynamics and polyelectrolyte properties of large biological polymers, in particular, <u>polynucleotides</u> and <u>nucleic acids</u> are being studied by <u>electric-field induced dichroism</u> and <u>birefringence</u> methods.</p> <p>The current research is a response to the fact that the knowledge of the <u>structural</u> effects of specific base-pair sequences on DNA translation and replication is still at a primitive stage. Only one or two biologically significant protein-DNA complexes from which such structural effects could be inferred have been crystallized and their structure determined. Using electro-optic birefringence and dichroism, it is now possible to quantitatively explore DNA structures in solution, albeit with less resolution than x-ray diffraction of crystals, but uninhibited by the problem of forming crystalline complexes. The two principal projects currently being pursued are the structural effects of the triplet sequence CAC/GTG and the flexibility of the A form of DNA.</p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01-DK-29007-18-LCP
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Structure and interaction of biomolecules		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <div style="display: flex; justify-content: space-between;"> <div style="width: 30%;">P.I. : Hideo Kon</div> <div style="width: 40%;">Research Chemist</div> <div style="width: 30%;">LCP-NIDDK</div> </div> <div style="display: flex; justify-content: space-between; margin-top: 10px;"> <div style="width: 30%;">Others: Yasunori Fukushima</div> <div style="width: 40%;">Visiting Fellow</div> <div style="width: 30%;">LCP-NIDDK</div> </div>		
COOPERATING UNITS (if any) Robert P. Blumenthal C DCBD Makoto Chikira Chuo University, Japan		
LAB/BRANCH Laboratory of Chemical Physics		
SECTION Section on Spectroscopy and Structure		
INSTITUTE AND LOCATION NIH, NIDDK, Bethesda, MD 20892		
TOTAL MAN-YEARS: 1.5	PROFESSIONAL: 1.5	OTHER: 0
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div style="width: 30%;"> <input type="checkbox"/> (a) Human subjects  <input type="checkbox"/> (a1) Minors  <input type="checkbox"/> (a2) Interviews         </div> <div style="width: 30%;"> <input type="checkbox"/> (b) Human tissues         </div> <div style="width: 30%;"> <input checked="" type="checkbox"/> (c) Neither         </div> </div>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>In this project, we aim at applying electron paramagnetic resonance (EPR) spectroscopy to probe structure-function relationships in biological systems and attempt to develop a new mode of application. Continuing the application of the flow-spin label EPR method developed in this laboratory, we have investigated the mechanisms of the loss of red blood cell (RBC) deformability due to Heinz body formation by phenylhydrazine (PHZ) and monomethyl hydrazine (MMH). We have shown by the uniquely sensitive method that deformability decrease starts prior to visible Heinz body formation, in contrast to the previously held notion that the entire inner surface of the cell must be covered with Heinz bodies for the cell to show deformability decrease. Simultaneously measured membrane fluidity, the motional freedom of membrane proteins using a fatty acid spin label, and the low temperature EPR measurement of hemoglobin Fe(III) ion demonstrated that in PHZ insult, the denatured hemoglobins bind first to the membrane protein band 3 and immobilize the protein. The rigid structure then expands as the PHZ concentration is increased, eventually lining the inner surface of the cell. On the other hand, with MMH the amount of denatured hemoglobin binding on the membrane is much less and the cell deformability loss is caused rather by the increased viscosity of intracellular hemoglobin probably by oligomer formation. Also, a collaborative spin label study (with R.P.B.) of red cell and influenza virus membranes is underway to probe the possible effect of preparation conditions, such as the presence or absence of <math>Mg^{2+}</math>, <math>Ca^{2+}</math>, or ATP on the membrane fluidity.</p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01-DK-29008-18-LCP
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Electric and molecular structural investigation		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.:	Ruth McDiarmid	Research Chemist LCP-NIDDK
Others:	Donald Jordan Aharon Gedaknen	IRTA Fellow LCP-NIDDK Special Volunteer LCP-NIDDK
COOPERATING UNITS (if any) Leo Klasinc, Rugjer Bošković Institute, Zagreb, Yugoslavia Yuri Panchenko, Moscow State University, Moscow, USSR		
LAB/BRANCH Laboratory of Chemical Physics		
SECTION Section on Spectroscopy and Structure		
INSTITUTE AND LOCATION NIH, NIDDK, Bethesda, MD 20892		
TOTAL MAN-YEARS: 2.1	PROFESSIONAL: 2.1	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>The optical and resonant multiphoton ionization spectra of cyclopentadiene have been studied. From the experimental spectra we were able to identify and characterize three Rydberg series leading to the first ionization potential of the molecule. One additional transition was observed that was not a member of any Rydberg series, had a different vibrational sub-structure than the Rydberg transitions, required two photons to excite, and was of <math>A_1</math> symmetry. The upper state of this transition was identified as the long-sought <math>2A_1</math> valence state.</p> <p>The structure around the <math>3p</math> Rydberg transition of <math>CS_2</math> was measured and analyzed. The controversial <math>-396\text{ cm}^{-1}</math> band was identified as the triplet component of this transition. The sequence and series bands of the transition were used to probe the geometry and electronic couplings of the upper state.</p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29009-16 LCP

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on sickle cell disease

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: William A. Eaton Medical Officer LCP-NIDDK

Others: James Hofrichter Research Chemist LCP-NIDDK  
Garrott W. Christoph Expert LCP-NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Macromolecular Biophysics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.5

PROFESSIONAL:

1.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The major aim of this work is to provide a quantitative description of the gelation of hemoglobin S that can be used for understanding the pathophysiology of sickle cell disease and the development of therapeutic agents. The delay time of hemoglobin S gelation plays a critical role in the pathophysiology of sickle cell disease. At physiological saturations with oxygen gelation takes place in the majority of sickle cells at equilibrium in vitro, but is prevented from occurring in vivo because the delay times are sufficiently long that most cells return to the lungs and are reoxygenated before gelation has begun. To extend this description a laser photolysis technique is being developed to measure the delay time, as a function of saturation on physiological time scales over a wide range of hemoglobin S concentrations and saturations. With these data it will be possible to provide a more complete description of gelation in vivo. The measurement of the delay time on single cells using the laser photolysis technique can also be used as a sensitive method to assess the potential efficacy of agents that are potential drugs for the treatment of sickle cell disease. Once this technique is automated, it will be possible to examine a large number of agents, to compare intracellular gelation and clinical severity, and to follow changes in intracellular gelation in patients on various therapeutic protocols.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01-DK-29010-17-LCP																		
PERIOD COVERED October 1, 1988 to September 30, 1989																				
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Conformation and electronic structure of biological molecules																				
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">P.I.: William A. Eaton</td> <td style="width: 33%;">Medical Officer</td> <td style="width: 33%;">LCP-NIDDK</td> </tr> <tr> <td colspan="3"> </td> </tr> <tr> <td>Others: James Hofrichter</td> <td>Research Chemist</td> <td>LCP-NIDDK</td> </tr> <tr> <td>Eric R. Henry</td> <td>Research Physicist</td> <td>LCP-NIDDK</td> </tr> <tr> <td>Anjum Ansari</td> <td>Visiting Associate</td> <td>LCP-NIDDK</td> </tr> <tr> <td>Colleen M. Jones</td> <td>IRTA</td> <td>LCP-NIDDK</td> </tr> </table>			P.I.: William A. Eaton	Medical Officer	LCP-NIDDK				Others: James Hofrichter	Research Chemist	LCP-NIDDK	Eric R. Henry	Research Physicist	LCP-NIDDK	Anjum Ansari	Visiting Associate	LCP-NIDDK	Colleen M. Jones	IRTA	LCP-NIDDK
P.I.: William A. Eaton	Medical Officer	LCP-NIDDK																		
Others: James Hofrichter	Research Chemist	LCP-NIDDK																		
Eric R. Henry	Research Physicist	LCP-NIDDK																		
Anjum Ansari	Visiting Associate	LCP-NIDDK																		
Colleen M. Jones	IRTA	LCP-NIDDK																		
COOPERATING UNITS (if any) Maurizio Brunori and Massimo Coletta, University of Rome, Italy; Andrea Mozzarelli and Gian Luigi Rossi, University of Parma, Italy.																				
LAB/BRANCH Laboratory of Chemical Physics																				
SECTION Section on Macromolecular Biophysics																				
INSTITUTE AND LOCATION NIH, NIDDK, Bethesda, MD 20892																				
TOTAL MAN-YEARS: 2.5	PROFESSIONAL: 2.5	OTHER:																		
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews																				
SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)  <div style="margin-top: 20px;"> <p>Time-resolved optical spectroscopy with nanosecond lasers and molecular dynamics calculations are being employed to investigate ligand rebinding and conformational changes in hemoglobin subsequent to photodissociation of the carbon monoxide complex. A sensitive spectrometer and novel methods of data analysis have been developed to measure the kinetics of protein conformational changes, which give rise to small spectral changes, in the presence of large spectral changes due to ligand rebinding. These methods have been used to determine kinetic parameters for geminate rebinding in hemoglobin in both the R and the T quaternary structures, to measure the rate of the R to T structural change, and to measure the kinetics of the tertiary conformational changes resulting from ligand dissociation in both quaternary structures.</p> <p>In a related project the oxygen binding curves of single crystals of hemoglobin in the T quaternary structure have been measured using a microspectrophotometer. These studies indicate that there is little or no cooperativity in the binding, confirming the most important prediction of the two-state allosteric model.</p> </div>																				

## DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

PROJECT NUMBER

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK-29011-18-LCP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The physics and chemistry of photoreception

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. :	William A. Hagins	Medical Officer	LCP-NIDDK
Others:	S. Yoshikami	Research Biologist	LCP-NIDDK
	F. M. Hagins	Guest Worker	LCP-NIDDK
	M. C. Foster	Research Physicist	LCP-NIDDK
	P. Ross	Research Chemist	LMB-NIDDK
	K. Spring	Research Med. Officer	LKM-NHI
	R. Tate	Computer Systems Analyst	CSL-DCRT

## COOPERATING UNITS (if any)

Sudhir Sadhu, Student Volunteer, Thomas Jefferson High School, Alexandria, VA

## LAB/BRANCH

Laboratory of Chemical Physics

## SECTION

Section on Membrane Biophysics

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

2.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Diffusion of fluorescent dyes injected by patch microelectrodes or by hydrolytic transfer of lipid-soluble dye precursors has been studied in outer segments of bullfrog retinal rods by image-intensified video microscopy. By comparing the longitudinal diffusion coefficients of the dyes with their diffusion in free solution, the geometric obstruction factor of the rhodopsin-bearing disks has been shown to be 20-fold or more. There is free lateral diffusion in the planes of the transverse disk membranes. 6-carboxyfluorescein, FURA-2, and BCECF all behave similarly, though with different diffusion coefficients.

Study by pyroelectric calorimetry of the heat transients produced during phototransduction by live frog retinas has continued. Effects of ionic substitution in the bathing solution and of inhibitors of cyclic nucleotide metabolism have been studied. A new calorimeter design offering improved sensitivity and permitting rapid measurements after changes in the fluid bathing the retina is under construction. A separate calorimeter for microliter enzyme samples has been designed and partially built.

A series of brominated compounds resistant to radiolysis in analytical electron microscopes has been synthesized for use as markers for water content and membrane potential within freeze-dried cells and subcellular compartments.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK-29012-19-LCP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The influence of molecular structure on chemical and biological properties

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I. : Norman E. Sharpless (Deceased) Research Chemist LCP-NIDDK

Others: Ralph G. Adams Research Physicist LCP-NIDDK  
William H. Jennings Research Physicist LCP-NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Chemical Physics

## SECTION

Section on Membrane Biophysics

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0

## PROFESSIONAL:

0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

INACTIVE

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29015-18-LCP

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Digital computer facilities for LCP and LMB

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: W.H. Jennings, Jr. Research Physicist LCP-NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Membrane Biophysics

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS:

0

PROFESSIONAL:

0

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The current laboratory computer system has been maintained. A new disk drive has been added to the Sun file server as well as several new work stations and a videocassette disk backup unit.

INACTIVE

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01-DK-29016-14-LCP																
PERIOD COVERED October 1, 1988 to September 30, 1989																		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Macromolecular dynamics and assembly reactions																		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">P.I.:</td> <td style="width: 33%;">James Hofrichter</td> <td style="width: 33%;">Research Chemist</td> <td style="width: 33%;">LCP-NIDDK</td> </tr> <tr> <td>Others:</td> <td>William A. Eaton</td> <td>Medical Officer</td> <td>LCP-NIDDK</td> </tr> <tr> <td></td> <td>Eric Henry</td> <td>Research Chemist</td> <td>LCP-NIDDK</td> </tr> <tr> <td></td> <td>Richard Lozier</td> <td>Research Chemist</td> <td>LCP-NIDDK</td> </tr> </table>			P.I.:	James Hofrichter	Research Chemist	LCP-NIDDK	Others:	William A. Eaton	Medical Officer	LCP-NIDDK		Eric Henry	Research Chemist	LCP-NIDDK		Richard Lozier	Research Chemist	LCP-NIDDK
P.I.:	James Hofrichter	Research Chemist	LCP-NIDDK															
Others:	William A. Eaton	Medical Officer	LCP-NIDDK															
	Eric Henry	Research Chemist	LCP-NIDDK															
	Richard Lozier	Research Chemist	LCP-NIDDK															
COOPERATING UNITS (if any)																		
LAB/BRANCH Laboratory of Chemical Physics																		
SECTION Section on Spectroscopy and Structure																		
INSTITUTE AND LOCATION NIH, NIDDK, Bethesda, MD 20892																		
TOTAL MAN-YEARS: <div style="text-align: center;">1.7</div>	PROFESSIONAL: <div style="text-align: center;">1.7</div>	OTHER:																
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews																		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p><u>Time-resolved absorption spectroscopy</u> is used to study the <u>dynamics</u> of <u>protein structural changes</u> subsequent to excitation with short laser pulses.</p> <p>A. Changes in the <u>tertiary</u> and <u>quaternary</u> structure of <u>human hemoglobin</u> (HbA) have been observed following the <u>photodissociation of carbon monoxide</u> from the hemes. The kinetics of <u>ligand rebinding</u> to the photoproduct molecule have been measured for both the R and T quaternary states. The results show that the <u>binding rate is reduced at least 30-fold</u> in the T quaternary structure within 20 ns after the iron-ligand bond is broken.</p> <p>B. Conformational changes have been observed and assigned in Trout I hemoglobin. The conformational dynamics of this molecule closely parallel those observed for HbA, with at least <u>biphasic tertiary relaxation</u> occurring on the <u>50 ns-2 us</u> timescale and a <u>quaternary relaxation</u> occurring at about <u>30 us</u> at room temperature. These processes have been assigned from experiments at various levels of photolysis, and the temperature dependence of the R-T relaxation rate has been studied.</p> <p>C. The photocycles of <u>bacteriorhodopsin</u> (bR), a photoactive proton pump have been investigated. Analysis of the spectra of the photoproducts of dark-adapted bR, which is an equilibrium mixture of two species, one of which contains the 13-cis and other the all-trans isomer of retinal, show that only <u>two cycles are initiated from these equilibrium species</u> at room temperature. Our studies of the all-trans cycle show evidence for <u>back reactions</u> in the steps which occur in less than 1 ms. Our results also suggest that a linear sequence of reversible steps is a sufficient description of the slower steps in the cycle.</p>																		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01-DK-29017-10-LCP
PERIOD COVERED: October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Spectroscopic investigation of membrane lipids and models		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)		
P.I.:	Ralph G. Adams	Research Physicist LCP-NIDDK
Others:	Ira W. Levin	Research Chemist LCP-NIDDK
COOPERATING UNITS (if any) Sherwin Strauss (FDA)		
LAB/BRANCH Laboratory of Chemical Physics		
SECTION Section on Molecular Biophysics		
INSTITUTE AND LOCATION NIH, NIDDK, Bethesda, MD 20892		
TOTAL MAN-YEARS:	1.5	PROFESSIONAL: 1.5 OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) Using integrated vibrational intensity techniques, our continuing research into the role of dipalmitoylphosphatidyl choline (DPPC) in lung surfactant has been supplemented by the new approach of Fourier transform (FT) Raman spectroscopy. FT Raman data thus far corroborates the findings obtained from conventional Raman spectroscopy as previously reported in 1988. It is yet too early to make conclusions about the dominant lipid/protein interactions in lung surfactant. However, the ability to obtain Raman spectra of native material, free from an overwhelming fluorescence signal, demonstrates that FT Raman techniques can provide an important new biophysical tool.		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01-DK-29019-09-LCP
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Theoretical studies on the dynamic aspects of macromolecular function		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I. :	A. Szabo	Research Chemist LCP-NIDDK
Others:	X. Zhou	Research Fellow LCP-NIDDK
COOPERATING UNITS (if any) R. W. Pastor, Biophysics Laboratory, FDA; B. R. Brooks, Division of Computer Research and Technology; R. Zwanzig, LCP-NIDDK; N. Agmon, Hebrew University		
LAB/BRANCH Laboratory of Chemical Physics		
SECTION Section on Molecular Biophysics		
INSTITUTE AND LOCATION NIH, NIDDK, Bethesda, MD 20892		
TOTAL MAN-YEARS: 1.5	PROFESSIONAL: 1.5	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)  <p> <u>Fluorescence quenching</u> studies of fluorophores attached to <u>proteins</u> or embedded in <u>membranes</u> can provide useful information concerning the nature of the dynamics in these systems. A variety of theories based on the <u>diffusive</u> model of microscopic dynamics that are required to interpret such experiments were investigated, the relationship among them was clarified and their range of validity was assessed. It was shown that a unified description of both the <u>steady state</u> and <u>transient</u> fluorescence intensity can only be given when the nonradiative lifetime of a quencher-fluorophore pair explicitly depends on the interparticle distance. Although fluorescence quenching is one of the simplest chemical reactions in solution, one is still faced with a many particle dynamical problem that cannot be solved exactly in general. A novel <u>density expansion</u>, analogous to the <u>virial expansion</u> for equilibrium properties, has been derived for the time dependence of the fluorescence intensity. This advance allows one to systematically obtain corrections to existing theories that based on various simplifying assumptions and should prove useful in bridging the gap between existing theory and experiment. Finally, the accuracy of various <u>Langevin</u> and <u>molecular dynamics</u> algorithms, that are used in computer simulations, has been investigated. This error analysis allows one to make a reasoned choice between algorithms and suggests specific strategies that should be used to optimally calculate the temperature, pressure and energy from trajectories.         </p>		

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK-29020-05-LCP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Nuclear magnetic resonance: new methods and molecular structure determination

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Ad Bax Visiting Scientist LCP-NIDDK

Others:	Rolf Tschudin	Electronics Engineer	LCP-NIDDK
	Dominique Marion	Visiting Associate	LCP-NIDDK
	Lewis Kay	Special Volunteer	LCP-NIDDK
	Mitsuhiko Ikura	Visiting Associate	LCP-NIDDK
	Hong Ha	Biological Laboratory Aid	LCP-NIDDK
	Guang Zhu	Student Volunteer	LCP-NIDDK

## COOPERATING UNITS (if any)

Marius Clore, Angela Gronenborn, NIDDK/LCP, Dennis A. Torchia, NIDR/LBR;  
B. Brooks, DCRT; Neil Glaudemans, NIDDK, Laboratory of Chemistry.

## LAB/BRANCH

Laboratory of Chemical Physics

## SECTION

Section on Nuclear Magnetic Resonance

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4.9

## PROFESSIONAL:

2.1

## OTHER:

2.8

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

New methods have been developed that permit structure determination of monomeric proteins of up to about 20 kD. A three-dimensional NMR scheme has been developed and applied to the proteins staphylococcal nuclease (18 kD), calmodulin (16.7 kD) and interleukin-18 (17 kD). These experiments proved invaluable in removal of extensive overlap present in the standard two-dimensional NMR spectra. Virtually complete backbone proton resonance assignments have now been obtained for staph. nuclease and interleukin-18, whereas the backbone assignments for calmodulin have progressed to the 50% stage.

New techniques have been developed for measuring local backbone dynamics in proteins and these have been applied to staph. nuclease. An average order parameter of 0.86 is found for the NH bond vector, which in a cone model would correspond to a semiangle of 18°. No correlation is found between the order parameter and the type of secondary structure, although significant differences in the order parameter are measured along the polypeptide backbone. Large amplitude motions that occur on a slow time scale ( $\mu$ s-ms) are observed in the loop region of the protein, where the active site is located.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29021-04-LCP

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Conformation and dynamics of biological macromolecules

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: Eric R. Henry Research Physicist LCP-NIDDK

Others: William A. Eaton Medical Officer LCP-NIDDK  
James Hofrichter Research Chemist LCP-NIDDK  
Lionel P. Murray Staff Fellow LCP-NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Nuclear Magnetic Resonance

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS:

1.5

PROFESSIONAL:

1.5

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have extended our previous studies of the structural and ligand-binding dynamics of component I of trout hemoglobin. We have measured time-resolved optical absorption spectra of this protein following photodissociation of bound carbon monoxide over a wide range of temperatures and fractional dissociations of ligands. A global description of the temporal evolution of the spectrum of the photolyzed molecule for degrees of photodissociation ranging from 10% to 100% requires six exponential relaxations involving ligand rebinding and/or protein conformational changes. The first two relaxations involve both geminate rebinding of dissociated ligands and tertiary structural changes in photolyzed subunits of the protein. The third relaxation has been assigned to the quaternary conformational change of the protein from the R to the T structure, based on the dependence of the amplitude of the associated spectral change on the degree of photolysis. The last three relaxations involve bimolecular ligand rebinding concomitant with interconversion between the R and T states and are being analyzed in terms of kinetic models involving multiple protein conformations. We have also derived oxygen binding curves for single crystals of human hemoglobin from polarized optical absorption spectra of crystals at various oxygen pressures. We have observed that hemoglobin in the crystal binds oxygen essentially non-cooperatively and with very low affinity.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK-29022-02-LCP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structural studies of AIDS proteins by NMR

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. :	Angela M. Gronenborn	Visiting Scientist	LCP-NIDDK
	G. Marius Clore	Visiting Scientist	LCP-NIDDK
	Ad Bax	Visiting Scientist	LCP-NIDDK
Others:	Paul C. Driscoll	Visiting Fellow	LCP-NIDDK

## COOPERATING UNITS (if any)

Glaxo Institute of Molecular Biology, Geneva (Paul Wingfield, Steven Stahl); E. Appella (NCI)

## LAB/BRANCH

Laboratory of Chemical Physics

## SECTION

Section on Nuclear Magnetic Resonance

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

2.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input checked="" type="checkbox"/> (c) Neither
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Work has been initiated on a number of structural problems related to proteins derived from the HIV virus. These include tat, rev and nef, and proteins of the immune system, in particular interleukin-1 $\beta$  and interleukin-8. At this time, work is principally concentrated on obtaining sufficient material in a highly purified and active form for structural studies. Using 3D heteronuclear NMR complete assignments of backbone and NH protons of interleukin-1 $\beta$  have been obtained. The secondary structure of interleukin-8 has also been determined and it has been shown that interleukin-8 is a dimer in solution in which the interface is stabilized by an anti-parallel  $\beta$ -sheet.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-DK-29023-02-LCP

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Determination of Three-Dimensional Structures of Macromolecules in Solution by NMR

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	G. Marius Clore	Visiting Scientist	LCP-NIDDK
	Angela M. Gronenborn	Visiting Scientist	LCP-NIDDK
Others:	Paul Driscoll	Visiting Fellow	LCP-NIDDK
	Michael Nilges	Visiting Fellow	LCP-NIDDK
	Julie Forman-Kay	Guest Researcher	LCP-NIDDK
	Jim Omichinski	IRTA Fellow	LCP-NIDDK

COOPERATING UNITS (if any)

Glaxo Institute of Molecular Biology, Geneva (Paul Wingfield); NCI (Ettore Appella); University of South Dakota (Robert Peanasky); University of Uppsala (Alwyn Jones)

LAB/BRANCH

Laboratory of Chemical Physics

SECTION

Section on Nuclear Magnetic Resonance

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

TOTAL MAN-YEARS:

2.55

PROFESSIONAL:

2.55

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Work in this laboratory has been focussed on the determination of three-dimensional structures of macromolecules in solution by NMR. Methods are being developed to increase the precision with which structures can be determined, the molecular weight range of proteins that can be analyzed, and the efficiency of the computational methods used to determine the structures on the basis of the NMR data.

The solution structures of a number of small proteins have been determined. These include wild-type and the Lys-47→Glu putative active site mutant of hirudin, and the C-terminal domain of cellulase (CT-CBH I). Using a new method for obtaining stereospecific assignments based on a conformational data base search, together with iterative procedures in the structure determination, has enabled us to obtain much more precise structures than was heretofore possible. Thus, in the case of CT-CBH I, we were able to obtain a set of 41 calculated structures with an average atomic rms difference about the mean coordinate positions of  $0.33 \pm 0.04$  Å for the backbone atoms and  $0.52 \pm 0.06$  Å for all atoms.

Work is in progress on determining the solution structures of a number of other proteins. These include the DNA binding protein *ner* from phage Mu, human interleukin-1β, human interleukin-8, human thioredoxin and a trypsin inhibitor from *Ascaris*. In the case of human interleukin-1β and *ner*, extensive use of 3D heteronuclear NMR has been made to resolve problems associated with spectral overlap and proton chemical shift degeneracy.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK-29024-02-LCP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Design of Agents for Fluidizing HIV Viron Membrane

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. : Ira W. Levin

Research Chemist LCP-NIDDK

Others: E. Neil Lewis

Visiting Associate

LCP-NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Chemical Physics

## SECTION

Section on Molecular Biophysics

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

## TOTAL MAN-YEARS:

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

INACTIVE

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  201-DK-29025-01-LCP
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Investigations of Macromolecular Structures in Solution by NMR		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.:	Angela M. Gronenborn G. Marius Clore	Visiting Scientist Visiting Scientist  LCP-NIDDK LCP-NIDDK
Others:	Paul Driscoll Michael Nilges Julie Forman-Kay Jim Omichinski	Visiting Fellow Visiting Fellow Guest Researcher IRTA Fellow  LCP-NIDDK LCP-NIDDK LCP-NIDDK LCP-NIDDK
COOPERATING UNITS (if any) Glaxo Institute of Molecular Biology, Geneva (Paul Wingfield); NCI (Ettore Appella); University of South Dakota (Robert Peanasky); University of Uppsala (Alwyn Jones)		
LAB/BRANCH Laboratory of Chemical Physics		
SECTION Section on Nuclear Magnetic Resonance		
INSTITUTE AND LOCATION NIH, NIDDK, Bethesda, MD 20892		
TOTAL MAN-YEARS 2.55	PROFESSIONAL 2.55	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard, unexpanded type. Do not exceed the space provided.)  <p>The objective of the overall research in this laboratory is centered on achieving as complete a description as possible for the structures of peptides, proteins, nucleic acids and their complexes in solution, principally by NMR spectroscopy. At present particular emphasis is placed on developing approaches which will allow the investigation of larger systems as well as increase the precision with which these solution structures can be obtained.</p> <p>Structures for several proteins have been determined and analyzed. These include the C-terminal domain of cellulase (CT-CBH I), wild-type hirudin and the Lys-47→Glu putative active site mutant thereof. Using a new method for obtaining stereospecific assignments based on a conformational data base search, together with iterative procedures in the structure determination, has enabled us to obtain much more precise structures than was heretofore possible. Thus, in the case of CT-CBHI, we were able to obtain a set of 41 calculated structures with an average atomic rms difference about the mean coordinate positions of <math>0.33 \pm 0.04</math> Å for the backbone atoms and <math>0.52 \pm 0.06</math> Å for all atoms.</p> <p>Work is in progress on a number of other proteins. These include the DNA binding protein ner from phage Mu, human interleukin-1<math>\beta</math>, human interleukin-8, human thioredoxin and a trypsin inhibitor from Ascaris. In the case of human interleukin-1<math>\beta</math> and ner, extensive use of 3D heteronuclear NMR has been made to resolve problems associated with extensive spectral overlap and proton chemical shift degeneracy.</p>		

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK-29026-01-LCP

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

NMR and Other Spectroscopic Studies of Molecular Structure

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. : Edwin D. Becker Research Chemist

LCP-NIDDK

## COOPERATING UNITS (if any)

Sophisticated Instruments Facility, Indian Institute of Science, Bangalore, India; Centre for Cellular and Molecular Biology, Hyderabad, India

## LAB/BRANCH

Laboratory of Chemical Physics

## SECTION

NMR Section

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A. Collaborative studies with C. L. Khetrpal and others at the Indian Institute of Science use nuclear magnetic resonance (NMR) methods to study molecules oriented in liquid crystals. Recent theoretical and experimental investigations have focused on samples spun rapidly in the magnetic field about an axis inclined to the field at the "magic angle" of 54.7 degrees. It has been shown that the intensities of the spinning sidebands so generated can be used to determine the signs of the order parameters of the molecules. The sign of this fundamental parameter is difficult to determine in other ways.

B. A new analysis of the fundamental vibrations of p-benzoquinone has been carried out, using recently acquired Raman spectroscopic data, together with infrared spectra published from this Laboratory many years ago. In spite of the relative simplicity of this molecule, its electronic and vibrational spectral assignments continue to generate interest and some controversy. The new analysis also takes into account several specific features that have become available in the last two years from other laboratories.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01-DK-29027-01-LCP
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Theoretical studies of dynamical processes in chemical physics and biophysics		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) P.I. : Robert Zwanzig                      Research Chemist                      LCP-NIDDK		
COOPERATING UNITS (if any) R. W. Pastor, Biophysics Laboratory, FDA; N. Agmon, DCRT		
LAB/BRANCH Laboratory of Chemical Physics		
SECTION Section on Macromolecular Biophysics		
INSTITUTE AND LOCATION NIH, NIDDK, Bethesda, MD 20892		
TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  One area of research is concerned with practical models for the hydrodynamical interaction of a protein with its surrounding medium. These are needed both for better understanding of protein dynamics and for more efficient computer simulations. An effective anisotropic bead model has been developed and tested. A second area is concerned with many-body (or concentration) effects in diffusion controlled reactions in which all species may diffuse. The standard Smoluchowski theory was found to remain approximately valid when three-body effects were included. A third area involves the dynamics of diffusion in complex systems, having for example multi-dimensional potential surfaces or entropic barriers. Also the general utility, and the origin, of the Agmon-Hopfield scheme for ligand binding kinetics is being explored.		

ANNUAL REPORT OF THE LABORATORY OF BIOORGANIC CHEMISTRY  
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The research of the Laboratory is directed towards the introduction of new concepts, techniques and agents for the elucidation of the molecular nature of mechanisms controlling cell functions. Specific focus is placed on i) Development of selective agonists/antagonists for receptors controlling cyclic nucleotide formation, phospholipid metabolism and ion channel function; ii) The relationship between ion transport, phospholipid turnover and cyclic nucleotide generation and the delineation of agents with specific effects on macromolecules involved in these systems. iii) The isolation and structure elucidation of biologically active natural products and definition of the basis of their activity. iv) Effects of agents on ion channels and the development of radioactive ligands for modulatory sites in such channels. v) The nature of enzymes involved in formation and inactivation of neurotransmitters, hormones, and other modulatory substances, in particular the enzymes, catechol-O-methyltransferase, monoamine oxidase, adenylate cyclase and phosphodiesterases. vi) The fundamental mechanisms by which drugs and environmental chemicals are transformed in the body with emphasis on oxidative metabolism by cytochrome P-450 systems to generate active oxide metabolites that interact with macromolecules such as DNA and are metabolized by further oxidation, by hydrolysis and by conjugation with glutathione.

Some of the milestones for the Laboratory are as follows: i) Introduction of the adenine-prelabeling technique for study of cyclic AMP generation in intact cells; ii) The steroidal alkaloid batrachotoxin as a selective activator of sodium channels. iii) Histronicotoxin as a noncompetitive blocker of acetylcholine receptor channels and potassium channels. iv) Pumiliotoxins as myotonic and cardiotoxic alkaloids acting through sodium channels to elicit phosphoinositide turnover. v) N<sup>6</sup>-Substituted adenosines and 8-phenyl and 8-cyclohexylxanthines as selective and potent adenosine receptor agonists and antagonists suitable as radioligands for binding studies and for definition of A<sub>1</sub> and A<sub>2</sub> classes of receptors. vi) Forskolin as a specific and widely useful activator of adenylate cyclase. vii) Fluoronorepinephrines and analogs as selective alpha and beta-adrenergic agonists. viii) Production of antibodies to catechol-O-methyl transferase and their use in studying localization of this key catechol-metabolizing enzyme. ix) Definition of a relationship between receptor-activation of phosphoinositide breakdown; protein kinase C activation, and altered responses of cyclic AMP-generating systems. x) Maitoxin as a general activator for activation of phospholipase C and phospholipase A<sub>2</sub> in intact cells. xi) Discovery of the NIH shift of aryl substituents during P-450 catalyzed phenol formation and demonstration of arene oxides as intermediates. xii) Demonstration of oxidation-hydrolysis pathways that convert stereoselectively polycyclic aromatic hydrocarbons to ultimate diol epoxides that react with DNA. xiii) Discovery and formulation of the bay-region theory, which is predictive of the pathway for formation of reactive carcinogenic metabolites from polycyclic aromatic hydrocarbons.

The laboratory accomplishes its mission both through its own resources and through extensive collaborations with other laboratories both at NIH, at Universities, Museums, and other institutes and in drug and chemical companies. Such collaborations can involve sharing of expertise on syntheses, isolations, analyses and biological testing and field work to obtain sources of new natural products.

#### HONORS AND AWARDS

J.W. Daly

Japanese Society for the Promotion of Science Fellowship. Oct. 26 - Nov. 14, 1989.

Fourth International Kyoto Conference on New Aspects of Organic Chemistry. Plenary Lecture. Kyoto, Japan, Nov. 18, 1989.

International Society Neurochemistry Satellite Symposium on Adenosine Receptors in the Nervous System. Invited Lecture, Algarve, Portugal, April 20, 1989.

Sixth International Caffeine Workshop, Invited Lecture, Hong Kong, August 8, 1989.

International Meeting: Purine Nucleosides and Nucleotides in Cell Signalling: Targets for New Drugs. Coorganizer and Honorary Plenary Lecture, Sept. 17, 1989.

D.M. Jerina

Outstanding Alumni Achievement Award, Knox College, Galesburg, IL.

H. Yagi

100th Annual Meeting Japanese Pharmaceutical Society, Plenary Lecture, Nagoya, Japan, April 2, 1989.

#### SECTION ON PHARMACODYNAMICS

##### Pharmacologically Active Compounds from Amphibians and Other Natural Sources

Alkaloids from Amphibians. Australian myobatrachid frogs of the genus *Pseudophryne* contain two distinct classes of alkaloids. The pseudophrynamine class (3a-prenyl-pyrrolo[2,3-b]indoles) are unique to this genus of frogs, while the pumiliotoxin-A class (8-hydroxy-8-methyl-6-alkylidene-1-azabicyclo-[4.3.0]nonanes) also occur in dendrobatid frogs of the genera *Dendrobates*, *Epipedobates* and *Minyobates*, in ranid frogs of genus *Mantella* and in bufonid toads of the genus *Melanophryniscus*. All seven species of *Pseudophryne* examined contain both classes of alkaloids. The pseudophrynamines were the predominant class in both species from Western Australia, while all of the eastern species contained

significant amounts of both pseudophrynamines and pumiliotoxins. Structures have been proposed for several new alkaloids of the pseudophrynamine class. Synthesis of pseudophrynamines is in progress. The biological activity of synthetic pseudophrynamines will be investigated.

The structure of a 4-hydroxypiperidine 241D, isolated from a Panamanian poison frog, Dendrobates speciosus, was shown by synthesis to be (cis-cis) 2-methyl-6-nonyl-4-hydroxypiperidine. It is a potent inhibitor of carbamylcholine-elicited ion flux in pheochromocytoma cells. The synthetic approach involves a one step condensation of an  $\alpha,\beta$ -unsaturated ketone, an aldehyde and ammonia to yield a 2,6-disubstituted-4-piperidone, which can be converted through reduction to either a 2,6-disubstituted piperidine-4-ols and piperidines. A range of such compounds have been prepared for biological evaluation as nicotinic blockers.

An alkaloid 251F, isolated from the Colombian poison frog Minyobates bombetes, has been isolated. Mass spectral, nmr, and infrared analysis allows the proposal of a tentative tricyclic cyclopentanoquinolizidine structure containing three methyl and one  $\text{CH}_2\text{OH}$  substituents.

Exchange of hydrogens on carbons adjacent to nitrogen have been shown to occur using noble metal catalysts saturated with deuterium gas. The method can be used for exchange labeling and for structural analysis.

Dendrobatid alkaloids of various classes have been detected in skin extracts from bufonid toads of the genus Melanophryniscus of Argentina and Paraguay. Isolation of major components, which include unusual homopumiliotoxins, is in progress. A tricyclic alkaloid, previously known only from ladybug beetles, has been identified. Further alkaloids have been detected and characterized in extracts from dendrobatid frogs. Dendrobatid frogs reared in captivity do not produce alkaloids. Two possible mechanisms for lack of alkaloids are under investigation with captive-bred frogs. These are i) lack of environmental input necessary for expression of genome responsible for biosynthetic enzymes and ii) lack of dietary precursors. A population of a Panamanian poison frog, Dendrobates auratus, introduced into Hawaii fifty years ago, contains a markedly different profile of alkaloids than the parent population in Panama.

The binding of [ $^3\text{H}$ ]batrachotoxinin A benzoate (BTX-B) to a site on the sodium channel is antagonized by local anesthetics, straight chain alcohols, reserpines, and a variety of alkaloids. The inhibition appears competitive, but at least for local anesthetics probably involves binding at other sites, thereby causing stabilization of channels in closed conformations that do not bind [ $^3\text{H}$ ]BTX-B with high affinity.

A variety of dendrobatid alkaloids including histrionicotoxins, gephyrotoxins, piperidines, indolizidines, and decahydroquinolines are noncompetitive blockers of carbamylcholine-elicited sodium flux through nicotinic receptor-channels in pheochromocytoma cells. Certain of these alkaloids enhance desensitization of nicotinic receptors.

Pumiliotoxin B (PTX-B) and a variety of congeneric alkaloids and synthetic analogs stimulate sodium flux and phosphoinositide breakdown in guinea pig cerebral cortical synaptoneurosome. The effects of PTX-B and active congeners

and analogs on sodium flux in synaptoneurosome are markedly potentiated by scorpion venom. In neuroblastoma cells, PTX-B and active congeners have no effect on sodium flux unless synergized by  $\alpha$ -scorpion toxin or scorpion venom. Certain inactive congeners, lacking hydroxyl groups in the 6-alkylidene side chain, inhibit sodium flux elicited by PTX-B, scorpion venom, or the sodium channel activator batrachotoxin. Such inhibition appears different from inhibition by local anesthetics, since pumiliotoxins, unlike local anesthetics, have little or no effect on binding of [ $^3$ H]batrachotoxin-A benzoate to sodium channels. Thus, it appears likely that some "inactive" congeners bind to the PTX-B binding site, but do not activate sodium channels. It is proposed that PTX-B alkaloids can have agonist, antagonist or reverse agonist activity at a unique site on sodium channels.

## SECTION ON PHARMACODYNAMICS

### Pharmacology and Metabolism of Biogenic Amines and Related Compounds.

**Catechol-O-Methyl Transferase (COMT): Structural Studies.** Polysome immunoadsorption with a specific rabbit antiserum to a 23KD soluble rat liver COMT has been used to isolate a messenger RNA, which encodes a single polypeptide when translated *in vivo*. When translated, the purified message synthesizes a protein of 23KD and pI of 5.2. The isolated mRNA represents 0.46% of total rat liver polyadenylated message. The enrichment of this message has been used for the initiation of cloning with cDNA probes. A cDNA probe derived from a major peptide with the sequence AYVPVAPIXTDKINAADYA is being used in the search for clones.

**Adrenergic Agonists: Fluorine Derivatives of Biogenic Amines.** The 2- and 6-fluoroderivatives of the beta-adrenergic agonist N-t-butyl-3,4-dihydroxyphenoxypropanolamine have been synthesized and their potencies as beta-adrenergic agonists compared to the parent compound. In a manner similar to the effects of fluorine substitution on epinephrine, the agonist potency of the 2-fluoro derivative is 10-fold greater than the parent amine while the 6-fluoro derivative is virtually inactive. The affinities in beta-adrenergic receptor binding studies were in agreement with the agonist potencies in functional assays. These initial studies suggest that fluorine induced agonist specificities are not dependent on the presence of a benzylic hydroxyl group.

## SECTION ON PHARMACODYNAMICS

### Ion Channels, Receptors and Second Messengers in the Nervous System.

**Maitotoxin: A Unique Agent for Activation of Phospholipid Turnover.** Maitotoxin (MTX), a high molecular weight polyhydroxy polyether isolated from a marine dinoflagellate, causes contraction of muscle and release of neurotransmitters and hormones from nerves and secretory cells. Such actions have appeared due to activation by MTX of calcium channels and MTX has been proposed to be the most potent known activator of such channels with effects being seen at 100 pM. MTX has now been shown to be even more potent in stimulation or breakdown of phosphoinositides with effects being seen at 20 pM in a variety of cells, as measured by the accumulation of inositol phosphates. Phosphoinositide breakdown results in the formation of two second messengers; inositol trisphosphate, which

releases calcium from intracellular stores, and diacylglycerides, which activate protein kinase C. Activation of protein kinase C, elicited by MTX through formation of diacyl glycerides can either lead to sensitization or inhibition of cyclic AMP-generating systems.

MTX and a chemotactic peptide (fMLP) induce the formation of inositol phosphates in HL-60 cells differentiated with dibutyryl cAMP. The increase is rapid, but transient after fMLP stimulation, whereas the MTX-induced increase occurs at a slower rate and is sustained over time. In both cases increases in internal calcium, measured with fura-2, parallel the formation of inositol trisphosphate. MTX-mediated stimulation of inositol phosphate formation is inhibited in the absence of calcium, while the response to fMLP is not. The calcium ionophore ionomycin stimulates the formation of inositol phosphates and a rapid sustained increase in internal calcium in differentiated HL-60 cells. The magnitude of the inositol phosphate formation is smaller than that elicited by MTX. In undifferentiated HL-60 cells, neither fMLP nor ionomycin induce significant inositol phosphate formation, and the increase in calcium elicited by ionomycin is transient. In contrast, the effects of MTX on phosphoinositide breakdown and on calcium in undifferentiated cells are nearly identical to those elicited by MTX in differentiated cells. Through the use of the calcium chelator BAPTA, changes in intracellular calcium detectable with fura-2 were suppressed in differentiated HL-60 cells treated with fMLP or MTX. In these conditions fMLP and MTX still stimulate the generation of inositol phosphates. Guanyl nucleotides and calcium stimulate phospholipase C activity in membrane preparations from differentiated HL-60 cells, while MTX has no effect on membrane phospholipase C activity.

MTX increases both formation of [ $^3$ H]inositol phosphates from phosphoinositides and release of [ $^3$ H]arachidonic acid from phospholipids in pheochromocytoma PC12 cells. Formation of inositol phosphates is detected within 5 min of incubation, whereas release of arachidonic acid is not detected until 20 min. Stimulation of arachidonic acid release can be detected at 9 pM MTX, whereas 60 pM MTX is the threshold for detection of phosphoinositide breakdown. Organic and inorganic calcium channel blockers, except cadmium and to some extent manganese, have no effect on MTX-elicited phosphoinositide breakdown, while inorganic blockers, but not organic blockers (nifedipine, verapamil, diltiazem), inhibit MTX-stimulated arachidonic acid release. MTX-elicited influx of calcium-45 and an increase in internal calcium measured with fura-2 are markedly reduced by nifedipine. MTX-elicited phosphoinositide breakdown and arachidonic acid release are abolished or reduced, respectively, in the absence of extracellular calcium. The calcium ionophore A23187 has little or no effect alone, but in combination with MTX, A23187 inhibits phosphoinositide breakdown and enhances arachidonic acid release. The results suggest that different sites and/or mechanisms are involved in stimulation of calcium influx, breakdown of phosphoinositides and release of arachidonic acid by MTX.

Sodium Channels and Inositol Phosphate Formation. The stimulatory effects of pyrethroids on phosphoinositide breakdown in guinea pig synaptoneurosomes are similar to other agents that activate voltage-dependent sodium channels. Type II pyrethroids, such as deltamethrin and fenvalerate, are more potent in stimulating phosphoinositide breakdown and, at least for deltamethrin, more efficacious than type I pyrethroids, such as allethrin. The effects of type II pyrethroids are partially inhibited by the sodium channel blocker tetrodotoxin. The effects of type I pyrethroids are not affected by tetrodotoxin. Stimulation

of phosphoinositide breakdown by fenvalerate is not additive to stimulation elicited by sodium channel agents (batrachotoxin, scorpion venom and pumiliotoxin B), but is additive to stimulation with receptor agonists. Stimulation by allethrin is not additive to the stimulation elicited by sodium channel agents, but instead allethrin acts to reduce responses to both receptor agonists and sodium channel agent. A local anesthetic, dibucaine, which inhibits sodium channel activation, inhibits phosphoinositide breakdown induced by type II, while inhibiting type I responses only at much higher concentrations. Thus, type II pyrethroids appear to stimulate phosphoinositide breakdown in synaptoneurosomes in a manner analogous to other sodium channel agents, while type I pyrethroids elicit phosphoinositide breakdown by a different mechanism, probably not involving sodium channels.

## SECTION ON PHARMACODYNAMICS

### Adenosine Receptor Agonists and Antagonists

Adenosine Receptors:  $A_1$ -Adenosine receptors are inhibitory to adenylate cyclase in fat cell membranes, while  $A_2$ -adenosine receptors are stimulatory to adenylate cyclase in platelet and pheochromocytoma cell membranes. The binding of [ $^3H$ ]cyclohexyladenosine provides a measure of  $A_1$  receptors in brain membranes, while binding of [ $^3H$ ]N-ethylcarboxamidoadenosine can provide a measure of  $A_2$  receptors in striatal membranes. The assay of activity of a variety of compounds, including adenosine analogs, xanthines, pyrazolopyridines, imidazodiazopindiones, and 9-aryl-7-deazaadenines in these systems has led to the discovery of several highly potent and selective agonists and antagonists for  $A_1$  receptors, and a few somewhat selective antagonists for  $A_2$  receptors. However, the data also indicate that the  $A_1$  or  $A_2$  receptor of these model systems are not identical, so that apparent selectivity will vary dependent on which  $A_1$  and  $A_2$  system is being compared.

The effects of 8-phenyl and 8-cycloalkyl substituents on the activity of theophylline, caffeine, 1,3-dipropylxanthine, 1,3-dipropyl-7-methylxanthine, 3-propylxanthine, and 1-propylxanthine at  $A_1$  adenosine receptors of rat brain and fat cells and at  $A_2$  adenosine receptors of rat pheochromocytoma PC12 cells and human platelets have been compared. An 8-phenyl substituent has little effect on the activity of caffeine or 1,3-dipropyl-7-methylxanthine at adenosine receptors, while markedly increasing activity of theophylline, 1,3-dipropylxanthine, 1-isoamyl-3-isobutylxanthine, 1-methylxanthine, and 3-propylxanthine. 8-Phenyl-1-propylxanthine is potent ( $K_i = 20-70$  nM) at all receptors. A p-carboxy or p-sulfo substituent, which is introduced on the 8-phenyl ring to increase water solubility, in most cases decreases the activity and selectivity for the  $A_1$  receptor. Among the 8-p-sulfo analogues, only 8-(p-sulfophenyl)theophylline and 1,3-dipropyl-8-(p-sulfophenyl)xanthine are selective for the  $A_1$  receptors. 8-p-Sulfophenyl derivatives of caffeine, 1,3-dipropyl-7-methylxanthine, and 3-propylxanthine are somewhat selective for the  $A_2$  receptors. 8-Cycloalkyl substituents (cyclopentyl, cyclohexyl), markedly increase activity of caffeine and 1,3-dipropyl-7-methylxanthine at the  $A_2$  receptor. 8-Cyclohexylcaffeine is potent ( $K_i = 190$  nM) and selective for the human platelet  $A_2$  receptor, but is not as selective for the rat PC12 cell  $A_2$  receptor. Such  $A_2$  selectivity is in contrast to the marked  $A_1$  selectivity of 8-cycloalkyltheophyllines and 8-cycloalkyl-1,3-dipropylxanthines.

Various sulfur-containing analogs of 8-substituted xanthines remain active at  $A_1$  and  $A_2$  receptors. Generally the 2-thio-8-cycloalkylxanthines are at least as  $A_2$  selective as the parent oxygen analog, while 2-thio-8-aryl xanthines tend to be more potent at  $A_2$  receptors than the parent oxygen analogs. 6-Thio analogs are less active. Certain 2-thio-8-substituted xanthines are virtually specific for  $A_1$  receptors.

Pharmacological Activity of Xanthines: A variety of xanthines cause tracheal relaxation, an activity predictive of antiasthmatic potential. Structural analogs of caffeine, theophylline, and enprofylline were examined for their ability to relax carbamylcholine-stimulated guinea pig trachea in vitro. All caffeine analogs tested are more potent than caffeine ( $EC_{50} = 550 \mu M$ ) except the 8-p-sulfophenyl analog, which is virtually inactive, 1,3,7-Tripropylxanthine and 1,3,7-tripropargylxanthine are among the more potent analogs with  $EC_{50}$  values of 12 and 65  $\mu M$ , respectively. Increasing the polarity at the 1- or 3-position by substituting a propargyl group for an n-propyl group decreases relaxant activity, an effect not observed at the 7-position. The 8-cyclohexyl-, 8-cyclopentyl- and 8-phenyl-derivatives of caffeine are relatively potent ( $EC_{50} = 75 \mu M$ ). The theophylline analog, 1,3-di-n-propylxanthine is approximately two times more active than theophylline ( $EC_{50} = 162 \mu M$ ). 3-Isobutyl-1-methyl-xanthine ( $EC_{50} = 7 \mu M$ ) and 1-isoamyl-3-isobutylxanthine ( $EC_{50} = 37 \mu M$ ) are among the most potent tracheal relaxants. The 8-substituted theophylline analogs are weak or inactive relaxants except 8-cyclopentyl- and 8-cyclohexyltheophylline, which are more potent or as potent as theophylline. In contrast to enprofylline ( $EC_{50} = 56 \mu M$ ), 8-substituted enprofylline analogs are weak or inactive as relaxants with the exception of the 8-cyclohexyl analog. Potency of xanthines as tracheal relaxants is unrelated to potency as adenosine receptor antagonists and may reflect activity as phosphodiesterase inhibitors.

## SECTION ON OXIDATION MECHANISMS

### Enzymatic Oxidation of Drugs to Toxic and Carcinogenic Metabolites.

pH-Rate profiles for the hydrolysis of quinoline arene oxides were measured in 1:9 dioxane-water at 25 °C (0.1 M NaClO<sub>4</sub>) and compared with the parent carbocyclic compound naphthalene 1,2-oxide. Unlike naphthalene 1,2-oxide whose hydrolysis rate shows a first-order dependence on hydronium ion concentration ( $k_H$ ) below pH 5, rates for the quinoline 5,6- and 7,8-oxides show a  $k_H$  reaction (0.14 and 1.54 M<sup>-1</sup> s<sup>-1</sup> for quinoline 5,6- and 7,8-oxides, respectively), but plateau below pH 2-3. pH-Independent rate constants in the low-pH plateau are  $2.7 \times 10^{-5}$  and  $1.7 \times 10^{-5}$  s<sup>-1</sup> for the 5,6- and 7,8-oxides, respectively. On the basis of studies of the N-methyl cation of the 5,6-oxide ( $k_{obsd} < 4 \times 10^{-5}$  s<sup>-1</sup> at pH 1.8), it is concluded that the low-pH plateau is due to lack of reactivity for the N-protonated quinoline oxides. Also unlike naphthalene oxide, the quinoline oxides show a reaction with hydroxide ion ( $k_{OH}$  rate) that results in the formation of trans dihydriodols. The 100- to 1000-fold decrease in reactivity of the quinoline oxides, relative to naphthalene oxide in the pH range of 1-10, can be accounted for in terms of the presence of their ring nitrogens and the differences in the  $pK_a$  values of these nitrogens.

K-Region 5,6-oxides of chrysene, benz[a]anthracene, and benzo[c]phenanthrene undergo nucleophilic trans attack by tert-butylthiolate anion in aqueous

dioxane to give mixtures of regioisomeric thiol adducts. These adducts have been separated by HPLC, and the positions of thiol addition have been assigned by NMR and/or CD spectroscopy. Reaction of either one of a pair of regioisomeric adducts with boron trifluoride in ether gave the same mixture of aryl alkyl thioethers, as a result of sulfur migration, presumably via a common episulfonium ion intermediate. Under these acid conditions, both chrysene adducts gave exclusively 6-(tert-butylthio)chrysene, both benz[a]anthracene adducts gave predominantly (87%) 5-(tert-butylthio)benz[a]anthracene, and both benzo[c]phenanthrene adducts gave predominantly (90%) 5-(tert-butylthio)benzo[c]phenanthrene. We have observed for the first time that thiol adducts of K-region arene oxides also undergo a facile base-catalyzed elimination of water in the presence of sodium methoxide in THF. In contrast to the acid-catalyzed reactions, the reaction of each pure adduct under these basic conditions gave a single aryl alkyl thioether that resulted from dehydration without migration of the sulfur substituent. Reaction of several thiol adducts of benzo[c]phenanthrene 5,6-oxide in acid has been observed to yield benzo[c]phenanthrene. A proposed sulfenyl chloride intermediate from this reaction in hydrochloric acid has been trapped by its addition to cyclohexene. It is suggested that hydrocarbon formation occurs by attack of a nucleophile on the sulfur of a cationic intermediate.

The rates of reactions of the *cis* and *trans* (benzylic 7-hydroxyl group relative to epoxide oxygen) bay-region 7,8-diol 9,10-epoxides of benzo[a]pyrene (DE-1 and DE-2, respectively) in solutions containing human serum albumin (HSA) and a model system of micelles of the nonionic detergent Tween-80 have been determined as a function both of HSA or Tween-80 concentrations and of pH. The effect of increasing concentrations of both HSA and Tween-80 is to retard substantially the rates of reaction of DE-1 and DE-2 over the pH range 5-7. The rate data are consistent with a mechanism in which the diol epoxides physically associate with HSA or Tween-80, and the rates of reaction of the association complexes are reduced compared to those of free diol epoxides. The limiting rate constants for reaction of the diol epoxide-HSA and diol epoxide-Tween-80 complexes are dependent on pH. The rate data are best accommodated by a mechanism in which the complexes react by two competing pathways, one whose rate is proportional to hydronium ion activity, and the second whose rate is pH-independent. The spontaneous reaction of the (DE-2)-HSA complex results in approximately 10% covalent binding of diol epoxide to the protein, whereas the acid-catalyzed reaction of the complex results in significantly less covalent binding.

Mutations were induced in the *supF* gene of the pS189 shuttle vector by treatment with optically active benzo[c]phenanthrene (4R,3S)-diol (2S,1R)-epoxide in vitro and replication in human cells. Most of the mutations analyzed were transversions (86%), which principally consisted of similar numbers of A.T → T.A and G.C → T.A changes. The unusual susceptibility of A.T pairs to mutation by this chemical agent is consistent with its chemical reactivity toward adenine and argues that the mutations are targeted to the adducts formed. The central base in the sequences 5'-AGA-3', 5'-AAC-3', and 5'-GAG-3' was particularly susceptible to mutation. Twelve "hotspots" in the *supF* gene accounted for most mutations seen. Some of these hotspots differed from those found by others for racemic benzo[a]pyrene diol epoxide and, even when a hotspot was common, the mutagenic changes were not always the same. The cellular machinery that converts chemical damage to mutations must determine the mutational result to a large extent, but the findings show that the chemical agent itself plays a large role in determining

both the location and the nature of the mutations that arise. Additional binding studies of the four optically active benzo[c]phenanthrene diol epoxides to rat c-H-ras oncogene established significant differences in their sequence selectivities. Of particular interest was the finding that the identical stereoisomers from a different hydrocarbon, benzo[a]pyrene, displayed altered selectivity.

## SECTION ON OXIDATION MECHANISMS

### Mechanistic Enzymology of HIV Proteins.

A continuous optical assay for DNA polymerases, based upon changes in the circular dichroism (CD) spectrum upon elongation of the double-helical portion of template/primer complexes ( $\text{dA}_{40-60} \cdot \text{dT}_{20}$  or poly  $\text{rA} \cdot \text{dT}_{20}$ ) has been developed. DNA- and/or RNA-directed DNA polymerization catalyzed by the Klenow fragment of *E. coli* DNA polymerase I and reverse transcriptase from HIV-1 were monitored by following the increase in the absolute magnitude of the circular dichroism signal at 248 nm. The incorporation of 1 nmole of [methyl- $^3\text{H}$ ]TTP into the template/primer complex,  $\text{dA}_{40-60} \cdot \text{dT}_{20}$ , corresponds to a CD change of -1.5 mdeg at 248 nm. Values of  $K$  and  $V_{\text{max}}$  for the polymerization reaction catalyzed by both enzymes and measured by the CD assay were in close agreement with those measured by a standard radiochemical assay. The 3' to 5' exonuclease activity of the Klenow fragment, which degrades the double helical structure, could also be followed by monitoring the decrease in the absolute magnitude of the CD signal at the same wavelength. This CD assay allows the continuous measurement of the entire time course of either the polymerization or the degradation reaction with a single sample. Since it is rapid and simple, and uses readily available non-radioactive substrates, this assay should be particularly well suited to the screening of inhibitors for these enzymes.

The two diastereomeric 2',3' epoxy analogs of adenosine triphosphate, which have the epoxide group in the  $\alpha$  and the  $\beta$  orientations, have been prepared and are currently under investigation as possible inhibitors of HIV RT. 4-Thiothymidine triphosphate is a substrate for the Klenow fragment of *E. coli* DNA polymerase I in the presence of  $\text{dA}_{40-60} \cdot \text{dT}_{20}$  as a template/primer, and its enzymatic reaction produces an easily measured absorbance change at 340 nm. Of particular interest is the fact that this substrate, upon incorporation into a nucleic acid structure, should form a less strong hydrogen bond in a Watson-Crick duplex than does thymidine itself. The effect of this factor upon the kinetic behavior and extent of chain elongation by various DNA polymerizing enzymes will be investigated.

On the basis of structural studies from several laboratories it is now clear that the protease of HIV-1 contains aspartyl residues at the putative active site and bears a strong resemblance to the aspartyl proteases of Rous Sarcoma (RSV) and avian myeloblastosis (AMV) viruses. Unlike mammalian aspartyl proteases, these viral proteases are dimeric enzymes. We have developed a rapid and convenient spectrophotometric assay for the AMV protease based upon the specific cleavage of a chromogenic peptide substrate. This assay should be applicable to the protease of HIV-1 upon modification of the peptide sequence, and will be used in the investigation of potential inhibitors for these viral enzymes.

This project is designed to develop more convenient assay methods for HIV enzymes and to elucidate their mechanism of action through the use of substrate analogs and specific inhibitors. The implications for rational design of anti-HIV drugs are self-evident. Even if the specific inhibitors investigated do not have anti-HIV activity per se, knowledge gained concerning the active sites of HIV enzymes is expected to provide valuable guidance in the design of the next generation of mechanism-based inhibitors. The development of rapid, continuous optical assays for reverse transcriptase and retroviral proteases will facilitate greatly the screening of potential inhibitors for these important classes of viral enzymes.

## SECTION ON OXIDATION MECHANISMS

### Mass Spectrometry of Drugs, Metabolites and Natural Products.

**Fragmentation Pathways of Frog Alkaloids:** Amphibian skin has proven a rich source for a variety of unique alkaloids. Work has commenced on determining the mass spectral fragmentation pathways of some classes of frog alkaloids, making it possible to better predict the structure of unknown compounds, even when they are present in mixtures. A study on the pumiliotoxin-A class is now complete and the pathways have been determined. However, as these results are being obtained on a new type of instrument, an Ion-Trap mass spectrometer, a large effort has been necessary to understand the ion physics in this instrument. The nature of the "self-CI" is now understood and the MS/MS fragmentation of simple esters and ketones has been elucidated. Using this experience, work is also beginning on the determination of purity of optical isomers by mass spectrometry.

**Plasma Desorption Mass Spectrometry:** Plasma desorption mass spectrometry is a method particularly suited to the analysis of non-volatile and/or high molecular weight compounds and has found a wide range of uses from small organic molecules through to peptides, and also in the organometallic field. Analysis routines have been enhanced and the instrument is being modified to produce high resolution results through the incorporation of a reflectron to further extend its potential.

**Bioactive Molecules from South Pacific Marine Organisms:** The structure of several biologically active marine natural products has been elucidated. These compounds are being investigated for their antiviral, antibacterial and anti-AIDS potency.

**Chinese Medicinal Compounds:** China has a long history of the use of medicinal plants for cures of various illnesses, but the active compounds in these plants are generally unknown. The oil from the plant Magnolia officinalis, which has been used as an asthma cure, has been fully characterized. The structures of three new terpene esters from Premna fulva Craib have been elucidated, and some unusual alkaloids from another plant species are under investigation.

**Snake Pheromones:** In collaboration with LC, NHLBI, snake pheromones are being investigated. One part of the project is aimed at finding an attractant/repellent to be used in the control of the Brown Tree snake, which is causing serious social and economic problems to the people of Guam.

**Sample Load:** During the past year, approximately 1300 samples were submitted for analysis and a further 450 analytical runs were completed as part of the Ion-Trap MS project.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 31100-24 LBC

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Pharmacologically Active Compounds from Amphibians and Other Natural Sources

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	J.W. Daly	Chief	LBC, NIDDK
Others:	C.R. Creveling	Research Chemist	LBC, NIDDK
	T. Spande	Research Chemist	LBC, NIDDK
	M. Edwards	Chemist	LBC, NIDDK
	F. Gusovsky	Senior Staff Fellow	LBC, NIDDK
	H.M. Garaffo	Visiting Fellow	LBC, NIDDK
	Y. Nishizawa	Guest Worker	LBC, NIDDK

COOPERATING UNITS (if any) Tokuyama, Osaka City U., Osaka, Japan; Kanaoka, Hokkaido Univ., Sapporo, Japan; Erspamer, U. Roma, Rome, Italy; Myers, Am. Mus. Nat. History, NYC; Balt., MD; Aronstam, U. GA., Augusta, GA; Overman, U. CA, Irvine, CA; Rossignol, DuPont de Nemours and Co., Wilmington DE., Wisnieski, Nat. Aquarium at Balt., MD.

## LAB/BRANCH

Laboratory of Bioorganic Chemistry

## SECTION

Section on Pharmacodynamics

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

4.2

## PROFESSIONAL:

4.2

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Natural products have provided a wide range of biologically active agents, many of which have unique profiles of pharmacological activity and therapeutic potential. Over two hundred alkaloids have been identified in extracts from amphibian skins. These include batrachotoxins, which are potent activators of sodium channels, histrionicotoxins, which are noncompetitive blockers of nicotinic receptor channel complexes and of potassium channels, and pumiliotoxins, which have myotonic and cardiotonic activity due to inhibitory effects on closing of sodium channels. Pumiliotoxin B acts at a specific site on the sodium channel to increase sodium flux and augments the effects of other sodium channel agents, such as the scorpion toxins and brevetoxins. Indeed, in neuroblastoma cells, scorpion toxin is required for activity of pumiliotoxins. Certain pumiliotoxins appear to prevent the action of pumiliotoxin B and to perhaps actually inhibit activation of sodium channels. Activity of pumiliotoxins as activators or inhibitors of sodium channel function is strongly dependent on structure of a alkylidene side chain. A variety of dendrobatid alkaloids are noncompetitive blockers of nicotinic receptor channel complexes. The binding of a batrachotoxin analog to a site on sodium channels is antagonized, apparently through stabilization of closed conformations of channels, by local anesthetics, reserpines, aliphatic alcohols, and a variety of alkaloids. The biological activity of trace alkaloids from dendrobatid frogs, such as the azatricyclododecenes, a cyclopentanoquinolizidine, amidines and homopumiliotoxins, and of the tricyclic indole pseudophrynamines from myobatrachid frogs remain unknown. One amidine alkaloid of as yet unknown structure is a potent analgetic.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 31101-21 LBC
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Pharmacology and Metabolism of Biogenic Amines and Related Compounds		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) P.I.: C.R. Creveling                      Research Chemist                      LBC, NIDDK Others: J.W. Daly                      Chief                      LBC, NIDDK F. Gusovsky                      Senior Staff Fellow                      LBC, NIDDK		
COOPERATING UNITS (if any) Kirk, LC, NIDDK; Brossi, LAC, NIDDK; Grossman, U. Penn., Phil. PA.; Breakfield, Shriver Inst., Waltham, MA.; Inoue, Okayama U., Okayama, Japan.; Seamon, NCDB, FDA.; Guroff, BB, CH; Harrison, U. Penn., York, PA.; Thakker, Glaxo Inc., Res. Triangle, NC.		
LAB/BRANCH Laboratory of Bioorganic Chemistry		
SECTION Section on Pharmacodynamics		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>The chemistry, biochemistry, physiology, and pharmacology of biogenic amines, amino acid precursors and metabolic products, and various synthetic derivatives have been investigated. The general areas of interest include catechol-O-methyltransferase (COMT) and the effects of fluorine substitution on the properties of biogenic amines and amino acids. Studies specific to COMT include: 1) the primary sequence of peptides isolated from the 23KD form of rat liver soluble COMT, 2) COMT specific cDNA probes and identification of antibody-derived clones, 3) isolation of human placental COMT 4) immunohistochemical localization of COMT and catechol estrogens in estrogen sensitive tissues, 5) substrate specificity and reaction mechanism of COMT with isoquinoline derivatives as putative precursors of mammalian opioids and with the mono- and difluorine substituted epinephrines and dihydroxy-phenylserines. Studies on fluorine substituted compounds include a) the synthesis and determination of the adrenergic properties of 2- and 6-fluoro derivatives of epinephrine, alprenolol, and N-t-butyl dihydroxyphenoxypiprolamine; b) mechanism of toxicity of 6-fluoro-, 2,6-difluorophenylalanine and 6-fluoro- and 2,6-di-fluorotyrosine in cultured pheochromocytoma (PC12) cell lines, c) the localization of 5-fluoronorepinephrine in cultured bovine chromaffin cells by electron energy loss spectroscopy.</p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 31102-18 LBC																		
PERIOD COVERED October 1, 1988 to September 30, 1989																				
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Ion Channels, Receptors and Second Messengers in the Nervous System																				
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">P.I. J.W. Daly</td> <td style="width: 33%;">Chief</td> <td style="width: 33%;">LBC, NIDDK</td> </tr> <tr> <td>Others: F. Gusovsky</td> <td>Senior Staff Fellow</td> <td>LBC, NIDDK</td> </tr> <tr> <td>O. Choi</td> <td>Visiting Fellow</td> <td>LBC, NIDDK</td> </tr> <tr> <td>J.A. Waters</td> <td>Research Chemist</td> <td>LBC, NIDDK</td> </tr> <tr> <td>P. Holbrook</td> <td>NRC Fellow</td> <td>LBC, NIDDK</td> </tr> <tr> <td>W. Padgett</td> <td>Biologist</td> <td>LBC, NIDDK</td> </tr> </table>			P.I. J.W. Daly	Chief	LBC, NIDDK	Others: F. Gusovsky	Senior Staff Fellow	LBC, NIDDK	O. Choi	Visiting Fellow	LBC, NIDDK	J.A. Waters	Research Chemist	LBC, NIDDK	P. Holbrook	NRC Fellow	LBC, NIDDK	W. Padgett	Biologist	LBC, NIDDK
P.I. J.W. Daly	Chief	LBC, NIDDK																		
Others: F. Gusovsky	Senior Staff Fellow	LBC, NIDDK																		
O. Choi	Visiting Fellow	LBC, NIDDK																		
J.A. Waters	Research Chemist	LBC, NIDDK																		
P. Holbrook	NRC Fellow	LBC, NIDDK																		
W. Padgett	Biologist	LBC, NIDDK																		
COOPERATING UNITS (if any) Yasumoto, Tohoku Univ. Sendai, Japan; Rossignol, Dupont de Nemours, and Co., Wilmington, Del.																				
LAB/BRANCH Laboratory of Bioorganic Chemistry																				
SECTION Section on Pharmacodynamics																				
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892																				
TOTAL MAN-YEARS: 4.1	PROFESSIONAL: 3.1	OTHER: 1.0																		
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews																				
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Physiological functions are mediated by a variety of second messengers, including cyclic AMP and cyclic GMP. Ions, such as calcium, sodium, potassium, and magnesium, can serve after translocation through ion channels or by transport proteins as second messengers to cause activation of release processes, contractile proteins, adenylate and guanylate cyclase, phosphodiesterases, protein kinases, phospholipases, ATPases and other enzymes. Enzymatic hydrolysis of phospholipids also generates second messengers, such as arachidonate, which serves as a precursor of prostanooids, diacylglycerides, which serve as activators of protein kinase C, and inositol phosphates, which serve as mobilizers of internal calcium ions. Receptors of various types and various toxins serve to modulate ion channels and generation of second messengers. Maitotoxin (MTX), isolated from a marine dinoflagellate, is a high molecular weight, water soluble, polyhydroxy polyether. MTX increases calcium flux in a wide range of cells. Such increases are blocked by channel blockers. In contrast, the calcium-dependent MTX-elicited increase in phosphoinositide breakdown, which occurs in all cell types, is not blocked by calcium channel blockers, nor is it prevented by chelation of internal calcium. It appears either that MTX requires calcium to bind to an extracellular site of action or that MTX elicits an influx of calcium to an activation site for calcium on phospholipase C within the membrane. Stimulation of phosphoinositide breakdown by MTX results in activation of protein kinase C and functional changes in cyclic nucleotide formation consonant in different cell types with activation of protein kinase C. MTX also stimulates a calcium-dependent formation of arachidonate through phospholipase A<sub>2</sub>. Activation of sodium channel in synaptoneurosomes triggers inositol phosphate formation. Type II pyrethroids, containing a cyano group, are more efficacious in activating sodium channels and in stimulating inositol phosphate formation than are type I pyrethroids. It would appear that type II pyrethroids stimulate inositol phosphate formation primarily through activation of sodium channels, while type II pyrethroids act through another mechanism.</p>																				

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  <div style="text-align: center;">Z01 DK 31104-21 LBC</div>
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Enzymatic Oxidation of Drugs to Toxic and Carcinogenic Metabolites		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.:	D.M. Jerina	Section Chief LBC, NIDDK
Others:	J. Sayer	Research Chemist LBC, NIDDK
	H. Yagi	Visiting Scientist LBC, NIDDK
	S.K. Balani	Guest Worker LBC, NIDDK
	A. Cheh	Research Chemist LBC, NIDDK
	D.R. Bushman	Staff Fellow LBC, NIDDK
	N.T. Nashed	Senior Staff Fellow LBC, NIDDK
	A. Chadha	Visiting Fellow LBC, NIDDK
COOPERATING UNITS (if any) A. Conney, Rutgers U. (Newark, NJ), W. Levin, Roche Inst. (Nutley, NJ); D. Whalen, Dept. of Chem., U. of MD (Catonsville); D. Boyd, Dept. of Chem., Queen's Univ. of Belfast (N. Ireland); D. Thakker, Center for Drugs and Biologics, FDA (Bethesda, MD); A. Dipple, FCRF (Frederick, MD).		
LAB/BRANCH Laboratory of Bioorganic Chemistry		
SECTION Section on Oxidation Mechanisms		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
8.5	7.5	1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>The primary goal has been the elucidation of the structures of reactive metabolites which are responsible for the carcinogenic, cytotoxic and mutagenic activity of drugs, polycyclic aromatic hydrocarbons, and other environmental chemicals. The approach taken consists of: i) synthesis of primary and secondary oxidative metabolites, ii) study of the metabolism of the chemicals with liver microsomes, as well as with purified and reconstituted cytochrome P-450 systems with and without epoxide hydrolase, iii) tests for mutagenicity of the synthetic metabolites, iv) elucidation of the roles of the cytochrome P-450 system and epoxide hydrolase in modulating the mutagenicity of these metabolites, v) determination of the carcinogenic activity of these compounds, vi) determination of the rates and products of reactions of arene oxides and diol epoxides with biopolymers and model compounds, and vii) search for agents capable of preventing the tumorigenic action of active metabolites. Current chemical studies have documented novel solvolytic reactivity for arene oxides at the 5,6- and 7,8-positions of quinoline which are from 100- to 1000-fold less reactive than their naphthalene 1,2-oxide counterpart. In addition, hydrolysis rates for the quinoline oxides become insensitive to acid concentration below pH 3. Urine from animals treated with polycyclic aromatic hydrocarbons that have K-regions contains a water soluble metabolite which liberates the hydrocarbon on HCl treatment. Based on the reactivity of model compounds, N-acetyl cysteine adducts of K-region arene oxides have been proposed as the hydrocarbon precursors via sulfenyl chloride intermediates. The solution lifetime of carcinogenic bay-region diol epoxide metabolites is substantially enhanced in the presence of hydrophobic proteins such as human serum albumin. Use of a shuttle vector has shown that a highly carcinogenic diol epoxide from benzo[c]phenanthrene caused mainly transversions which principally consisted of similar numbers of A.T → T.A and G.C → T.A changes. Studies of the covalent binding of four different optical isomers of the benzo[c]phenanthrene diol epoxides to c-H-ras oncogene showed wide divergence in their sequence selectivities.</p>		

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 31105-04 LBC

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Nicotinic and Muscarinic Acetylcholine Receptor Agonists.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	J.A. Waters	Research Chemist	LBC, NIDDK
Others:	J.W. Daly	Chief	LBC, NIDDK
	Y. Nishizawa	Guest Worker	LBC, NIDDK

## COOPERATING UNITS (if any)

A. Aronstom, Med. Coll., Georgia; T. Gund, Newark Coll. of Eng. & Chem., N.J.; C. Spivak, NIDA, Baltimore, MD; I. Stolerman, U. of London, England.

## LAB/BRANCH

Laboratory of Bioorganic Chemistry

## SECTION

Section on Pharmacodynamics

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

0

## CHECK APPROPRIATE BOXES)

☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Potent, semirigid nicotinic acetylcholine receptor agonists have been synthesized in order to better understand the acetylcholine receptor recognition sites. Numerous compounds, generally of the acetyl substituted piperidine and piperazine type, and bicyclic amines of the anatoxin-a type, have been prepared for structure-activity correlations. Computer assisted modeling studies have given minimum energy conformations, superimposability diagrams of the hydrogen bond acceptor and the cationic head onto the template, and electrostatic potentials at the van der Waals surfaces, providing additional information for a rational approach to the design of new, potent agonists. Isoarecolone (1-methyl-4-acetyl-1,2,3,6-tetrahydropyridine) methiodide is the most potent of these synthetic nicotinic agonists as shown in various assays: (i) Torpedo electric tissue (high density of nicotinic receptors), (ii) frog rectus abdominus muscle (neuromuscular receptors), (iii) rat pheochromocytoma PC12 cells (ganglionic receptors), and (iv) rat brain membranes (central receptors). Also, isoarecolone hydrochloride produced nicotine-like discriminative effects in rats. Isoarecolone methiodide is only moderately potent at muscarinic M1 receptors (rat brain) in comparison to acetylcholine and exhibits weak activity at M2 receptors (heart).

Nicotinic agonists and muscarinic agonists/antagonists may be useful in the treatment of cholinergic deficient diseases such as Alzheimer's disease, where reduced levels of acetylcholine, acetylcholine receptors and cholineacetyltransferase are found, and myasthenia gravis, where autoantibodies are directed to the main immunogenic region (MIR) of the alpha-subunit of the nicotinic receptor. A study of the effectiveness of isoarecolone salts in animal models of Alzheimer's disease (systemically and intracerebroventricularly) is in progress.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 31106-02 LBC
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Mechanistic Enzymology of HIV Proteins.		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.: D.M. Jerina Others: J.M. Sayer N.T. Nashed B.J.R. Forbes J.G. Baillon	Section Chief Research Chemist Senior Staff Fellow Staff Fellow Visiting Fellow	LBC, NIDDK LBC, NIDDK LBC, NIDDK LBC, NIDDK LBC, NIDDK
COOPERATING UNITS (if any) S. Broder, NCI.		
LAB/BRANCH Laboratory of Bioorganic Chemistry		
SECTION Section on Oxidation Mechanisms		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 2.6	PROFESSIONAL: 2.5	OTHER: 0.1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Methods of enzymology, chemical and enzymatic kinetics, and synthetic, physical and analytical chemistry are being used to develop novel agents targeted against the reverse transcriptase (RT) and protease enzymes of HIV-1. i) A convenient, continuous optical assay for RT and other DNA polymerases has been developed. This assay is based upon the increase in the absolute magnitude of the circular dichroism (CD) signal at 248 nm due to elongation of the double helical portion of a synthetic template/primer complex. The 3' to 5' exonuclease activity of the Klenow fragment of <u>E. coli</u> DNA polymerase I can also be followed by observing the decrease in the absolute magnitude of the CD signal at the same wavelength. Thus, this assay is potentially capable of measuring enzymatic catalysis of either polymerization or degradation of nucleic acid duplexes. ii) The two diastereomeric adenosine triphosphate analogs, which have an epoxide group in the alpha- and beta- orientation at the 2',3'-position of the sugar, have been prepared and are being investigated as possible inhibitors of RT. The interactions of RT with other nucleoside triphosphate analogs which may be either inhibitors or substrates of this enzyme are also under study. iii) A continuous spectrophotometric assay for viral aspartyl proteases, based on specific cleavage of a chromogenic peptide substrate, has been developed, using the protease of avian myeloblastosis virus which has sequence and structural similarities to the HIV enzyme. This assay is applicable to the HIV protease by changing the amino acid sequence of the chromogenic peptide. iv) Several phenolic and quinonoid compounds that are related to polycyclic aromatic hydrocarbons have been tested for anti-HIV activity in cell culture. To date the compounds tested have been either inactive, cytotoxic or both.</p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 31107-02 LBC
PERIOD COVERED October 1, 1988 to September 30, 1989.		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Mass Spectrometry of Drugs, Metabolites and Natural Products.		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) P.I.: L. Pannell Visiting Scientist LBC, NIDDK Others: D. Jerina Section Chief LBC, NIDDK Q-1. Pu Visiting Scientist LBC, NIDDK J. Daly Laboratory Chief LBC, NIDDK		
COOPERATING UNITS (if any) Johnson, Whittaker and White, LAC, NIDDK; Basile, LNS, NIDDK; Fales, Mason and Sokolowski, LC, NHLBI, NIH; Munro, Blunt and Brooker, Univ. Canterbury, NZ; Jacobson, LC, NIDDK; Shields and Povey, NCI; West, Univ. Illinois; Mauger, Medlantic Res. Found.; Ito, LTC, NHLBI; Guangxi Inst. Trad. Med. Pharm. Sci.		
LAB/BRANCH Laboratory of Bioorganic Chemistry		
SECTION Section on Oxidation Mechanisms		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 2.2	PROFESSIONAL: 2.2	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  This project was initiated in LBC to provide specialized mass spectrometry analysis, primarily but not exclusively of trace organic compounds isolated from biological systems. Over the past year, an Ion-Trap mass spectrometer and a Fourier Transform Infrared spectrometer have been introduced as invaluable tools for the routine use of laboratory personnel. A new high resolution mass spectrometer is currently being evaluated in order to provide high resolution gas chromatographic and peptide analysis. The joint development with LC, NHLBI, of Californium plasma desorption and Ion-Trap mass spectrometers for analysis of non-volatile high molecular weight compounds and complex natural products has provided invaluable new approaches to characterization of such compounds. Identification of pheromones from the skin of various snakes provides the basis for possible development of repellents and/or attractants for harmful snake species. The countercurrent chromatograph mass spectrometry interface has been adapted for trace component separations. Mass spectrometry has been utilized for the structural identification of a variety of biologically active marine natural products from South Pacific. Samples for mass spectral analysis derive from many facilities and researchers outside LBC.		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 31108-01 LBC
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Adenosine Receptor Agonists and Antagonists		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.	J.W. Daly	Chief LBC, NIDDK
Others:	L.E. Brackett	IRTA Fellow LBC, NIDDK
	M. Shamim	Guest Worker LBC, NIDDK
	C. Mueller	Guest Worker LBC, NIDDK
	I. Hide	Guest Worker LBC, NIDDK
COOPERATING UNITS (if any) Gustaffson, Karolinska Inst., Stockholm, Sweden; Ribeiro, Gulbenkian Inst., Portugal; Seales, U. Oklahoma, OK; Weir, Howard U., Wash., D.C.; Olsson, U. So. Florida, Tampa, FL; Neumeyer, Res., Biochem. Inc., Natick, MA; Eger, U. Tubingen, W. Germany; Jacobson, LC, NIDDK.		
LAB/BRANCH Laboratory of Bioorganic Chemistry		
SECTION Section on Pharmacodynamics		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 1.2	PROFESSIONAL: 1.2	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Adenosine regulates a wide range of physiological functions through interaction with at least two major classes of adenosine receptors. The A<sub>1</sub> class of adenosine receptors is inhibitory to adenylate cyclase, while the A<sub>2</sub> class is stimulatory to adenylate cyclase. Subclasses of adenosine receptors also occur. Some of these are inhibitory to calcium channels, some are stimulatory to potassium channels, some can activate guanylate cyclase, some can modulate phospholipid, while those of smooth muscle cause relaxation through a poorly defined mechanism. Structural analogs of adenosine and various xanthine antagonists have been developed as radioligands and as potent and/or selective agonists or antagonists for investigation of adenosine receptors and effector systems in membranes, cells, tissues and whole organisms. A variety of caffeine and theophylline analogs and other heterocycles, including pyrazolopyridines, imidazodiazopindiones and 9-aryl-7-deazaadenines are antagonists at both A<sub>1</sub>- and A<sub>2</sub>-classes of adenosine receptors, measured either versus binding of radioactive ligands or versus effects on adenylate cyclase. Many are selective for A<sub>1</sub> receptors, but in addition several have been identified as A<sub>2</sub> selective. The potencies of theophylline, caffeine and enprofylline analogs as tracheal relaxants, an activity predictive of antiasthmatic effects, correlates not with adenosine receptor blockade, but rather with inhibition of phosphodiesterases.</p>		

Annual Report of the Laboratory of Molecular Biology  
National Institute of Diabetes and Digestive and Kidney Diseases

The Laboratory of Molecular Biology has as its principal goal the understanding of biological processes at the molecular level. The research program involves application of theoretical and experimental methods to a wide variety of problems in molecular genetics, regulation of gene expression in eukaryotes, mechanisms of DNA replication, nucleic acid and protein structure, bioenergetics and transport properties of biological molecules. These include studies of enzyme and immunoglobulin structure by X-ray diffraction, investigations of polynucleotide chemistry, structure and interactions by spectroscopic methods, studies by calorimetry of proteins and nucleic acids, studies on the molecular mechanisms of for establishing and maintaining stable states of gene expression during embryogenesis, studies of the organization of DNA and proteins within the eukaryotic nucleus, studies of the effects of supercoiling on biological activity and protein-DNA interaction, as well as theoretical analyses of mechanisms of microtubule assembly, inter membrane diffusion of lipids and muscle contraction. There is increased interest in more direct studies of biological processes. These investigations include studies of the rearrangements that lead in vivo to the formation of assembled immunoglobulin genes, of the process of DNA replication in both prokaryote and eukaryotes, of the regulatory proteins that control expression of certain eukaryotic genes, of the effect of molecular crowding in biochemical systems, of non heritable antibiotic resistance and of the mechanism of genetic recombination. Significant progress has been made in all of these areas during the past year.

#### Enzyme Structure

The bifunctional enzyme complex tryptophan synthase from Salmonella typhimurium has been subject to extensive structural refinement. X-ray data sets have been measured for several site directed mutants. This enzyme converts indole glycerol phosphate and serine to tryptophan in two steps. The most striking result is the demonstration of a tunnel linking the two active sites through which the product of the first reaction (indole) can diffuse to the active site of the second enzyme.

Additional inhibitor studies have been carried out on the aspartyl proteinase, Rhizopuspepsin. These enzymes form a superfamily which contain the mammalian enzymes pepsin, renin and cathepsin D as well as the retroviral proteinases, including that from HIV-1. These inhibitor studies provide a basis for understanding the general mechanism of action of these enzymes.

### Three-Dimensional structure of Proteins of the Immune System

The structure of two antibody-antigen complexes have been determined and the structures refined. These are the monoclonal antibody HyHEL-5 Fab complexed to lysozyme and the monoclonal antibody Hy-HEL10 Fab complexed to a different part of the lysozyme surface. The refined data confirm the remarkable complementarity of the interacting surfaces of the antibody and antigen, and demonstrate the almost complete exclusion of water from the interface.

The crystal structure of an anti-galactan antibody has been completely refined to high resolution thus providing a basis for understanding the mode of binding to carbohydrate antigen. Also preliminary investigations have resulted in the crystallization of the Fab of an antibody, OKT4A, that recognizes the CD4 antigen on T-helper cell and blocks the binding of the AIDS virus.

### Studies on the Mechanism of Genetic Recombination

The mechanisms involved in the transposition-replication reaction of the bacteriophage Mu provide a model system for understanding the enzymatic steps involved in DNA rearrangement and insertion reactions. A critical step in the Mu transposition reaction is a pair of DNA strand transfers which generate an intermediate DNA molecule with a branched structure. Efficient formation of this intermediate requires Mu A, Mu B and E. coli HU proteins along with ATP and  $Mg^{++}$ . The Mu A protein binds to the Mu end DNA sequences on the donor DNA and makes a pair of single strand cuts to expose the 3' ends of the Mu sequence. This cleaved donor DNA with associated protein is an active intermediate which completes the DNA strand transfer by using a DNA molecule which is bound by Mu B protein as the target. The Mu B protein, which is an ATPase, selectively stimulates the utilization of intermolecular target DNA molecules which do not carry Mu end sequences. The Mu B protein dissociates selectively from the DNA molecule to which the Mu A protein is bound in a process that depends on the hydrolysis of ATP.

The N-terminal domain of the Mu A protein has been identified as binding to the Mu operator sequence, while the Mu end sequences are recognized by a separate internal domain. The N-terminal domain of the Mu A protein and the donor DNA sequence at the mu operator, while essential for the formation of the high order protein-DNA complex involved in recognition of the relative orientation of the two Mu end sequences, are dispensable from later reaction steps after the cleavages are made at the end of the Mu sequence. This finding has significant biological implications in the decision-making process of Lytic vs. lysogenic modes of Mu growth.

### Studies of Immunoglobulin Gene Rearrangement

Analysis of the various products of V(D)J recombination in lymphoid cells has shown that nucleotide insertions are not restricted to junctions between coding segments. Contrary to previous belief, insertions in the reciprocal junctions (signal joints) are quite common.

Taken together with our earlier finding of "hybrid joints" that fuse signal end to coding ends, these results show that signal joints and coding joints are recombined in ways more similar than had been thought.

A systematic study of the signal sequences has shown that only a subclass of the frequently conserved heptamer and nonamer sequences is necessary for recombination. These data are not consistent with the model that implicates base pairing between the signals, suggesting that it is probably protein-protein binding that brings the sites together for rearrangement.

#### Studies of Functions Involved in Genetic Recombination

In a continuing study of the structure and function of DNA gyrase, we are engaged in identifying the ATP-binding site of the B subunit of the enzyme. The affinity-labeling ATP analog, adenylyl-pyridoxal phosphate, is found to attach specifically and stoichiometrically to GyrB and to give essentially complete inhibition of activity. This inhibitor forms a Schiff base with a lysine which is in the process of being located in the protein.

#### Studies on the Mechanism of Retroviral Integration

Integration of a DNA copy of a retroviral genome into a chromosome of an infected cell is an essential step for normal viral replication. The objectives of this project are to analyze the detailed molecular mechanism of this DNA integration reaction and to develop simple cell-free integration systems that may be used to screen for potential anti-retroviral drugs that block this step of the replication cycle.

We have previously demonstrated, with Moloney murine leukemia virus (MoMLV) as a model system that DNA integration occurs by joining the 3' ends of the viral DNA to the 5' ends of a staggered cut made in the target DNA. Subsequently we developed an in vitro system that accomplishes this reaction. The DNA substrate for integration (mini-MoMLV) is a linear plasmid with ends that mimic the ends of authentic unintegrated MoMLV DNA. The viral proteins necessary for integration are provided as disrupted MoMLV virions. Inclusion of a cytoplasmic extract of uninfected cells in the reaction mixture greatly enhances the efficiency of integration. With this cell free system as the assay, we have determined that the MoMLV IN protein is the only viral protein that is essential for integration. This protein has been cloned and expressed in the Baculovirus system and has been purified to greater than 90% homogeneity. The cloned MoMLV IN protein substitutes for disrupted MoMLV virions as the source of viral proteins in our in vitro integration reaction.

The detailed mechanistic information we have obtained concerning the MoMLV DNA integration reactions are now being developed to study the integration of HIV. We have cloned and expressed the HIV IN protein and integration of mini-HIV DNA mediated by the HIV IN protein has been detected in preliminary experiments.

## Chromatin Structure and Function

We have continued our studies of chromatin structure in the neighborhood of expressed genes, making use of the globin gene family in chicken erythrocytes as a model system. We have made progress in understanding the detailed mechanisms by which the expression of these genes is regulated during development. We have focussed attention on the novel erythroid - specific factor, which we have named Eryf1. We have already shown that there is a binding site for this factor in regulatory regions near each member of the  $\alpha$ - and  $\beta$ -globin families, suggesting that Eryf1 may play a major role as a general "switch" factor in erythroid development. We have now cloned and analyzed the cDNA for Eryf1, and shown that its mRNA is present only in erythroid cells. Our data show that Eryf1, like many regulatory factors, is a "finger" protein. As a contrast to the general stimulation of globin gene expression provided by Eryf1, we have studied two additional factors, the Pal protein and GCS protein, that play a different role in development. The adult  $\beta$ -globin promoter contains binding sites for each of these, immediately adjacent to one another. By examining the binding of these factors as a function of developmental stage, coupled with expression studies, we have discovered a gene-specific regulatory mechanism that appears to be involved in specific shut-down of  $\beta$ -globin synthesis late in development. Developmental regulation of these genes seems to have a hierarchical organization, with general factors like Eryf1 acting on all members, and special factors like Pal and GCS factor to modulate expression of individual genes.

The promoter of the  $\epsilon$  globin gene has been sequenced and putative control regions are being analyzed by in vitro footprinting and gel mobility shift assays. The extracts used for these analyses are derived from erythrocytes obtained at different stages of development spanning the time when the  $\epsilon$  gene is fully active to the adult chicken, when all genes are totally inactive. These regions of differential binding within the  $\epsilon$  promoter are also being analyzed for functional activity by linkage to a reporter gene and subsequent transfection into primary erythrocytes.

## Developmental Regulation of Gene Expression

Our work has focussed on the molecular mechanisms responsible for establishing and maintaining stable states of gene expression during vertebrate embryogenesis. Progress has been achieved in three key areas. First, we have demonstrated that interactions between proteins binding to the promoter elements of a gene will have a central role in regulating transcription. Secondly, we have extended our understanding of the role of chromatin structure in preventing transcription factors from associating with genes. This work employs two novel approaches: curved DNA elements have been used to position a nucleosome relative to gene regions of defined function and the hydroxyl radical reagent has been used to provide new information regarding the structure of DNA in a nucleosome. Finally, we have used in vitro replication systems derived from Xenopus eggs to demonstrate that a competition exists between transcription complex assembly and chromatin assembly on replicating DNA.

Moreover we have demonstrated that conformational transitions in chromatin structure can have a dominant effect on differential gene activity. This confirms and extends our earlier demonstration of the specific and dominant repression of genes driven by histone H1 mediated transitions in chromatin structure.

The significance of this work is that it provides direct evidence for an active and central role of chromatin structure in causing the sequestration of the regulatory regions of eukaryotic genes. This role may pervade all regulated processes involving DNA in the eukaryotic nucleus e.g. replication, transcription, recombination, viral latency etc.

#### Nonheritable Antibiotic Resistance

We have previously found that salicylate and acetylsalicylate affect the regulation of several outer membrane protein genes in *E. coli*. This results in an altered outer membrane with reduced amounts of Ompf protein. As a consequence the outer membrane is found to be less permeable to various antibiotics and the salicylate grown cells show increased resistance to diverse antibiotics. We have now found that salicylate increases the sensitivity of these bacteria to various positively charged antimicrobials including many aminoglycosides. In contrast, bacteria carrying the kanamycin resistance determinant of Tn5 become more resistant to kanamycin in the presence of salicylate. Since these effects are seen *in vitro* at concentrations of salicylate that could be found in patients on high doses of aspirin, they could complicate the course of antibiotic therapy in such patients.

#### Influences of Macromolecular Crowding on Biochemical Systems

The high concentrations of macromolecules within cells will change the properties of a variety of biochemical reactions through excluded-volume effects. We have considered several methods of correcting parameters determined under conventional dilute solution conditions to conditions more closely approximating the highly volume-occupied conditions within cells. The first method is largely computational: the macromolecular composition within cells is represented as a distribution of hard spherical particles and scaled particle theory is used to calculate the desired activity coefficient for a test sphere of arbitrary size. There are obviously major assumptions made in such a calculation. As an independent test, we are developing an experimental assay for excluded volume in complex mixtures like cell extracts. The assay is based on distribution properties of macromolecules in the phosphate/polyethylene glycol two-phase partition system described by Albertson. The assay has yielded estimates of excluded volumes in solutions of individual proteins that are consistent with known properties of the proteins. Cell extracts can be manipulated at *in vivo* concentrations in the assay system. It remains to be seen if the assay is accurate enough with mixtures of macromolecules to estimate excluded volume effects in cell extracts.

### Thermal Measurements of Biomolecular Systems

A differential scanning calorimeter has been used to redetermine the thermal melting behavior of the DNA dodecamers containing purine-purine mismatch base pairs. We have established from the long extrapolations of the pre-transition and the post-transition base line that there is essentially no change in heat capacity in the vicinity of the transition temperature. This significant result establishes from a thermodynamic point of view that the thermally induced strand dissociation is a simple two-state process.

A computer program has been written (with A. Shrake) for simulation of the excess heat capacity profile of a multi-liganded macromolecule undergoing a reversible thermally induced transformation between native and denatured states. For both the native and denatured forms, provision is made for the introduction of an arbitrary number of ligand binding constants; each with their unique temperature variation. The concentration, molecular weight, transition temperature and the changes in enthalpy and heat capacity accompanying denaturation of the unliganded macromolecule are also entered into the calculation. At each temperature the degree of advancement of the denaturation reaction,  $\alpha$ , is calculated and the multi-ligand equilibria recomputed. The excess heat capacity is obtained from the temperature derivative of  $\alpha$ .

### Mammalian DNA Replication, Regulation and Amplification

Our investigation of the effect of the DNA sequence  $(GA)_n \cdot (CT)_n$  on DNA replication has continued. We have confirmed previous results that suggested these sequences slow fork progression by use of a new technique of two dimensional electrophoresis. We have extended previous results to demonstrate that slowing occurs irrespective of sequence orientation.

We are directly testing the role of  $(GA)_n \cdot (CT)_n$  sequences in amplification.

We have concluded an analysis of sequences thought to be enriched for mammalian replication origins and report them to be enriched for (i) a particular transcription regulatory element, (ii) AT-rich sequences (iii) matrix attachment regions and (iv) the ARS consensus sequence for S. cerevisiae but not S. pombe.

### Regulation of a gene expressed in Undifferentiated Cells

The Crypto Gene was found by serendipity in a screening of a cDNA library from human teratocarcinoma cells. The gene is expressed only in undifferentiated teratocarcinoma cells and shuts off on exposure of the cells to retinoic acid which induces differentiation. The gene is expressed in mouse teratocarcinoma cells and behaves similarly. The sequence of the gene looks remarkably like the growth factor EGF, but is clearly distinct from that factor in its expression. When placed under the control of an RSV promoter, crypto gene expression results in the

transformation of certain but not all cell types. We are currently investigating the role of the gene in differentiation.

### Statistical Thermodynamics of Protein and Polynucleotide Systems

Statistical mechanics was used to derive the binding of a ligand to a one-dimensional lattice in the presence of another ligand which compete with the first ligand. The binding isotherm equations derived for the system were then used to analyze the competitive binding data of myosin subfragment-1 and caldesmon to actin.

### Energy Conversion in Biology

A number of different topics have been studied in the general field of free energy transduction and biophysics of biological systems. The most important areas in which progress has been made are the study of redistribution of charge carriers near an energized membrane, the time-dependent assembly of microtubule on a bare nucleating site, video microscopic studies of virus-cell fusion, a theoretical study on the use of self-quenching fluorophores in virus-cell fusion kinetics, and the diffusion of lipids or lipid-like molecules between the membranes of a fusing virus-cell complex.

### Chemical and Structural Investigations of Nucleic Acids and Related Molecules.

We have investigated the binding of the antitumor antibiotics netropsin and distamycin to poly d(2NH<sub>2</sub>A-T) by UV and CD spectroscopy to obtain information on their known binding specificity to AT regions of DNA. The generally accepted explanation in the literature is that specificity is due to steric interference of the G-NH<sub>2</sub> group with CH of the drugs. The prediction was that 2-NH<sub>2</sub> of 2-NH<sub>2</sub>A would have a similar effect and prevent binding. We find, however, that both drugs bind as well to our modified polymer as to poly d(A-T), showing that the close contact is not present in the former case and suggesting that it is not the key element of binding specificity in natural DNA.

The conformation of dACATCGATGT, which contains recognition sites for the restriction endonucleases Taq I and Cla I, was studied by 2-D NMR. Estimates of scalar coupling constants and sugar pucker for all of the residues were obtained by  $\omega_1$ -scaled COSY and NOE experiments. The glycosidic torsion angles were determined and estimates of the backbone torsion angle,  $\epsilon$  obtained.

Modeling studies of our proposed structure for poly isoguanylic acid were carried out and suggested that a ramp of base pairs can be generated in three dimensions. All of the electronegative atoms except N7 are hydrogen bonded. The covalent chains are right handed, and the ramp of bases is left handed.

We have measured infrared spectra of a GC helix in the strongly absorbing solvent water and obtained unambiguous isotopic evidence for band assignments in the double bond region for the first time. Vibrational coupling of G and C carbonyl stretching bands through hydrogen bonds of the base pairs was also demonstrated.

The techniques of birefringence and photochemical electric dichroism have been used to analyze the changes in DNA structure of the 5S RNA gene of Xenopus borealis in the presence of zinc or spermidine. The 5S RNA gene of sea urchins has also been analyzed and an alternative structure observed that is associated with the oligopurine tracts within the regulatory region.

#### Replication, Recombination and Repair of Microbial DNA

Studies on DNA replication of plasmid ColE1 have been continued. Transcripts (RNA II) that start 555 nucleotides upstream of the replication origin by RNA polymerase form a hybrid with the template DNA. The hybridized transcript is cleaved by ribonuclease H at the origin and used as the primer for leading strand synthesis of DNA polymerase I. Primer formation is regulated by a plasmid-specified small RNA (RNA I). Synthesis of RNA I starts 445 base pairs upstream of the replication origin, proceeds in the direction opposite to that of RNA II synthesis, and terminates near the initiation site of RNA II synthesis. This anti-sense RNA binds to RNA II and inhibits formation of the secondary structure of RNA II that is necessary for primer formation. Primer formation is also regulated by a 63-amino acid protein specified by the plasmid. The protein called Rom binds to a very unstable initial complex made between RNA I and RNA II and thus enhances the inhibitory action of RNA I. Inhibition of primer formation by RNA I in the presence or absence of the protein determines the copy number of a plasmid in a cell and the incompatibility between related plasmids.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 33000-23 LMB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of Functions Involved in Genetic Recombination

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Martin Gellert	Chief, Section on Metabolic Enzymes	LMB/NIDDK
Others: James K. Tamura	Guest Worker	LMB/NIDDK
Mary H. O'Dea	Research Chemist	LMB/NIDDK

COOPERATING UNITS (if any)

Dr. G. Zaccai, Institut Max Von Laue-Paul Langevin, Grenoble, France  
Dr. A. Maxwell, University of Leicester, Leicester, U.K.  
Mr. S. Krueger, University of Maryland, College Park, MD

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Metabolic Enzymes

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

2.25

PROFESSIONAL:

2.25

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The ATP-binding site of the B subunit of DNA gyrase has been labeled with the affinity reagent adenylyl-pyridoxal phosphate, which binds to a lysine residue.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 33001-5 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of Immunoglobulin Gene Rearrangement

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Martin Gellert	Chief, Section on Metabolic Enzymes	LMB/NIDDK
Kiyoshi Mizuuchi	Chief, Section on Genetic Mechanisms	LMB/NIDDK
Others: Joanne Hesse	Research Chemist	LMB/NIDDK
Michael Lieber	Guest Worker	LMB/NIDDK
Joseph Menetski	IRTA	LMB/NIDDK
David Brown	Pratt Fellow	LMB/NIDDK

COOPERATING UNITS (if any) Dr. Moshe Sadofsky LMB/NIDDK  
Dr. Susanna Lewis, California Institute of Technology  
Dr. Melvin Bosma, Fox Chase Center for Cancer Research,  
Philadelphia, PA

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Section on Metabolic Enzymes

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4.5

## PROFESSIONAL:

4.5

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Analysis of the various products of V(D)J recombination has shown that nucleotide insertions are not restricted to junctions between coding segments. Contrary to previous belief, insertions in the reciprocal junctions (signal joints) are quite common. Thus signal joints and coding joints have more common features than expected.

A systematic mutational study of the signal sequences has shown that only a subclass of the frequently conserved heptamer and nonamer sequences is necessary for recombination. Furthermore, the results are not consistent with the prevalent model that requires base pairing between the signals.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 33002-03 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effects of DNA Supercoiling on the Topological Properties of Nucleosomes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: Martin Gellert

Chief, Section on Metabolic Enzymes

LMB/NIDDK

Gary Felsenfeld

Chief, Section on Physical Chemistry

LMB/NIDDK

Others: David Clark

Visiting Fellow

LMB/NIDDK

Mary H. O'Dea

Research Chemist

LMB/NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Section on Metabolic Enzymes

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.25

## PROFESSIONAL:

0.25

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Methods are being developed for studying the structure of nucleosomes on highly supercoiled DNA.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 KD 33006-11 LMB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on the Mechanism of Genetic Recombination

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Kiyoshi Mizuuchi, Chief, Section on Genetic Mechanisms LMB/NIDDK  
Others: K. Adzuma Visiting Associate LMB/NIDDK  
R. Craigie Visiting Scientist LMB/NIDDK  
M. Mizuuchi Visiting Associate LMB/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Section on Genetic Mechanisms

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

3

PROFESSIONAL:

3

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The major objective of this project is to uncover the enzymatic steps involved in various genetic rearrangement reactions and to study the mechanism of action of the enzymes involved. We are particularly interested in gene rearrangements caused by the transposon family of movable genetic elements. The mechanisms of the transposition-replication reaction of bacteriophage Mu is studied under this project as a model system.

A critical step in the Mu transposition reaction is a pair of DNA strand transfers which generate an intermediate DNA molecule with a branched structure. Efficient formation of this intermediate requires Mu A, Mu B and E. coli HU proteins along with ATP and  $Mg^{++}$ . The Mu A protein binds to the Mu end DNA sequences on the donor DNA and makes a pair of single strand cuts to expose the 3' ends of the Mu sequence. This cleaved donor DNA with associated proteins is an active intermediate which completes DNA strand transfer by using a DNA molecule which is bound by Mu B protein as the target. The Mu B protein, an ATPase, selectively stimulates the utilization of intermolecular target DNA molecules which do not carry Mu end sequences. The Mu B protein dissociates selectively from the DNA molecule to which Mu A protein is bound in a process that depends on hydrolysis of ATP. Kinetic aspects of this energy transduction system are studied.

Mu A protein possesses two separate sequence specific DNA binding domains. The N-terminal domain binds to Mu operator site away from the Mu ends and helps assembly of high order protein DNA complex necessary at the initiation of Mu DNA strand transfer reaction. This domain is dispensable from later reaction steps once the donor DNA is cleaved at each end of the Mu sequence.

The intermediate DNA molecules can be converted into cointegrates by DNA replication or into simple inserts by nucleolytic cleavages and gap repair. Both of these resolution pathways can be carried out using an E. coli cell extract and do not require Mu proteins.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 34001-24 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chromatin Structure and Function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Gary Felsenfeld, Chief, Section on Physical Chemistry LMB/NIDDK

## OTHERS:

David Clark, Visiting Fellow	LMB/NIDDK	Catherine Lewis, Staff Fellow	LMB/NIDDK
Todd Evans, Staff Fellow	LMB/NIDDK	Mark Minie, Staff Fellow	LMB/NIDDK
Hannah Gould, Expert	LMB/NIDDK	Joanne Nickol, Research Chemist	LMB/NIDDK
Takeshi Kimura, Visiting Fellow	LMB/NIDDK	Marc Reitman, Med. Staff Fellow	LMB/NIDDK
Joseph Knezetic, Guest Researcher	LMB/NIDDK	Cecelia Trainor, IRTA	LMB/NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Section on Physical Chemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

5.8

## PROFESSIONAL:

5.8

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- |   |  |   |
|---|--|---|
| <input type="checkbox"/> (a) Human subjects | <input type="checkbox"/> (b) Human tissues | <input checked="" type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors        |  |   |
| <input type="checkbox"/> (a2) Interviews    |  |   |

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have continued our studies of chromatin structure in the neighborhood of expressed genes, making use of the globin gene family in chicken erythrocytes as a model system. We have made progress in understanding the detailed mechanisms by which the expression of these genes is regulated during development. We have focussed attention on the novel erythroid - specific factor, which we have named Eryf1. We have already shown that there is a binding site for this factor in regulatory regions near each member of the  $\alpha$ - and  $\beta$ -globin families, suggesting that Eryf1 may play a major role as a general "switch" factor in erythroid development. We have now cloned and analyzed the cDNA for Eryf1, and shown that its mRNA is present only in erythroid cells. Our data show that Eryf1, like many regulatory factors, is a "finger" protein. As a contrast to the general stimulation of globin gene expression provided by Eryf1, we have studied two additional factors, the Pal protein and GCS protein, that play a different role in development. The adult  $\beta$ -globin promoter contains binding sites for each of these, immediately adjacent to one another. By examining the binding of these factors as a function of developmental stage, coupled with expression studies, we have discovered a gene-specific regulatory mechanism that appears to be involved in specific shut-down of  $\beta$ -globin synthesis late in development. Developmental regulation of these genes seems to have a hierarchical organization, with general factors like Eryf1 acting on all members, and special factors like Pal and GCS factor to modulate expression of individual genes.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 34002-25 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Enzyme Structure

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	David R. Davies, Chief, Section on Molecular Structure	LMB/NIDDK
Others:	Gerson H. Cohen, Research Chemist	LMB/NIDDK
	Craig Hyde, Senior Staff Fellow	LMB/NIDDK
	Eduardo Padlan, Visiting Scientist	LMB/NIDDK
	T.N. Bhat, Visiting Scientist	LMB/NIDDK
	Kevin Parris, IRTA Fellow	LMB/NIDDK

## COOPERATING UNITS (if any)

Edith W. Miles, Research Chemist

LBP/NIDDK

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Section on Molecular Structure

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.75

## PROFESSIONAL:

2.75

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- |   |  |   |
|---|--|---|
| <input type="checkbox"/> (a) Human subjects | <input type="checkbox"/> (b) Human tissues | <input checked="" type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors        |  |   |
| <input type="checkbox"/> (a2) Interviews    |  |   |

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

1) The crystal structure of the bifunctional enzyme complex tryptophan synthase has been subjected to several refinement procedures. X-ray-diffraction data have been collected and analyzed for several mutants prepared by site-directed mutagenesis.

2) Several x-ray data sets have been measured for the native and inhibited aspartyl proteinase, rhizopuspepsin.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 34003-21 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Three-Dimensional Structure of Proteins of the Immune System

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	David R. Davies, Chief, Section on Molecular Structure	LMB/NIDDK
Others:	Gerson H. Cohen, Research Chemist	LMB/NIDDK
	Enid W. Silverton, Research Chemist	LMB/NIDDK
	Eduardo A. Padlan, Visiting Scientist	LMB/NIDDK
	T.N. Bhat, Visiting Scientist	LMB/NIDDK
	Christina J. Brown, Visiting Fellow	LMB/NIDDK
	Boaz Shaanan, Visiting Scientist	LMB/NIDDK

## COOPERATING UNITS (if any)

Sandra Smith-Gill, NCI; Cornelis P. J. Glaudemans, NIDDK; Jay Unkeless, Rockefeller University

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Section on Molecular Structure

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

5

## PROFESSIONAL:

5

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

## Summary:

- 1) The structure of HyHEL-5 Fab-lysozyme complex has been completely refined in two crystal forms.
- 2) The structure of HyHEL-10 Fab-lysozyme complex has been completely refined.
- 3) The structure of the anti-galactan J539 Fab has been completely refined.
- 4) Crystals of the complex of J539 Fab with Gal, Gal-3 and Gal-4 have been obtained.
- 5) Crystals of the Fab of the monoclonal OKT4A antibody, that interferes with the binding of HIV-1 gp120 to CD4, have been obtained.
- 6) Crystals of the alpha-subunit of the IgG Fc receptor have been obtained.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 35000-25 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chemical and Structural Investigations of Nucleic Acids and Related Molecules

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: H. Todd Miles, Chief, Section on Organic Chemistry LMB/NIDDK

Others:	F. B. Howard	Research Chemist	LMB/NIDDK
	J. Frazier	Research Chemist	LMB/NIDDK

COOPERATING UNITS (if any) Girjesh Govil TIFR, Bombay, India / Philip Ross LMB/NIDDK  
V. Sasisekharan Fogarty Scholar; Indian Instit. of Science, Bangalore, India  
Dipak Dasgupta Saha Institute of Nuclear Physics, Calcutta, India  
Nagarajan Patibiraman Naval Research Laboratory, Washington, D. C.

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Section on Organic Chemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

4.5

## PROFESSIONAL:

4.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have investigated the binding of the antitumor antibiotics netropsin and distamycin to poly d(2NH<sub>2</sub>A-T) by UV and CD spectroscopy to obtain information on their known binding specificity to AT regions of DNA. The generally accepted explanation in the literature is that specificity is due to steric interference of the G-NH<sub>2</sub> group with CH of the drugs. The prediction was that 2-NH<sub>2</sub> of 2-NH<sub>2</sub>A would have a similar effect and prevent binding. We find, however, that both drugs bind as well to our modified polymer as to poly d(A-T), showing that the close contact is not present in the former case and suggesting that it is not the key element of binding specificity in natural DNA.

The conformation of dACATOGATGT, which contains recognition sites for the restriction endonucleases Taq I and Cla I, was studied by 2-D NMR. Estimates of scalar coupling constants and sugar pucker for all of the residues were obtained by  $\omega_1$ -scaled COSY and NOE experiments. The glycosidic torsion angles were determined and estimates of the backbone torsion angle,  $\epsilon$  obtained.

Modeling studies of our proposed structure for poly isoguanlylic acid were carried out and suggested that a ramp of base pairs can be generated in three dimensions. All of the electronegative atoms except N7 are hydrogen bonded. The covalent chains are right handed, and the ramp of bases is left handed.

We have measured infrared spectra of a GC helix in the strongly absorbing solvent water and obtained unambiguous isotopic evidence for band assignments in the double bond region for the first time. Vibrational coupling of G and C carbonyl stretching bands through hydrogen bonds of the base pairs was also demonstrated.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK35050-18 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Replication, Recombination and Repair of Microbial DNA

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. Tomizawa Chief, Section on Molecular Genetics LMB/NIDDK

Others: Y. Eguchi Visiting Fellow

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Section on Molecular Genetics

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2

## PROFESSIONAL:

2

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Studies on DNA replication of plasmid ColE1 have been continued. Transcripts (RNA II) that start 555 nucleotides upstream of the replication origin by RNA polymerase form a hybrid with the template DNA. The hybridized transcript is cleaved by ribonuclease H at the origin and used as the primer for leading strand synthesis of DNA polymerase I. Primer formation is regulated by a plasmid-specified small RNA (RNA I). Synthesis of RNA I starts 445 base pairs upstream of the replication origin, proceeds in the direction opposite to that of RNA II synthesis, and terminates near the initiation site of RNA II synthesis. This anti-sense RNA binds to RNA II and inhibits formation of the secondary structure of RNA II that is necessary for primer formation. Primer formation is also regulated by a 63-amino acid protein specified by the plasmid. The protein called Rom binds to a very unstable initial complex made between RNA I and RNA II and thus enhances the inhibitory action of RNA I. Inhibition of primer formation by RNA I in the presence or absence of the protein determines the copy number of a plasmid in a cell and the incompatibility between related plasmids.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 36003-5 LMB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Nonheritable Antibiotic Resistance		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: J.L. Rosner Research Biologist LMB/NIDDK Others: M. Aumercier Visiting Fellow LMB/NIDDK		
COOPERATING UNITS (if any) Foreign: Maria Persico, Senior Scientist, Int. Lab. of Gen & Biophysics Naples, Italy LSB/NIDDK, Research Chemist(J.D. Foulds) Children's Hospital, Philadelphia,PA, Pediatric Physician(M.Zasloff)		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Section on Microbial Genetics		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 2.25	PROFESSIONAL: 2.25	OTHER:
CHECK APPROPRIATE BOXES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)   <p>We previously described the effects of SAL on increasing the resistance of <u>E. coli</u> and <u>S. typhimurium</u> to antibiotics such as B-lactams, chloramphenicol, nalidixic acid and tetracycline. We have now found that salicylate increases the <u>sensitivity</u> of these bacteria to various positively charged antimicrobials including many aminoglycosides(kanamycin, kasugamycin, streptomycin, tobramycin, gentamicin), bleomycin and cadmium salts. Salicylate may cause these effects by increasing the membrane potential of the cells. In contrast, bacteria carrying the kanamycin-resistance determinant of Tn5 become <u>more resistant</u> to kanamycin in the presence of salicylate. Since these effects are seen in vitro at concentrations of salicylate (2.5mM) that may be found in certain patients on high doses of aspirin, they could complicate the course of antibiotic therapies in such patients.</p> <p>A strong synergism between two antibiotic peptides isolated from the frog, <u>Xenopus laevis</u>, has been found. Bacteria treated with low concentrations of magainin and PGLa are killed much more efficiently than when treated with either peptide alone.</p>		

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 36051-21 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mammalian DNA Replication, Regulation and Amplification

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Robert G. Martin, Chief, Section on Microbial Genetics LMB/NIDDK

Others: B. S. Rao Visiting Fellow LMB/NIDDK

G. Persico Visiting Scientist LMB/NIDDK

M. Reitman Senior Staff Fellow LMB/NIDDK

## COOPERATING UNITS (if any)

Foreign: M. Zannis-Hadjopoulos, McGill Cancer Center, Montreal, Canada  
G. Kaufmann, Tel Aviv University, Tel Aviv, Israel  
H. Manor, Technion University, Haifa, Israel

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Section on Microbial Genetics

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3.5

## PROFESSIONAL:

3.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Our investigation of the effect of the DNA sequence  $(GA)_n \cdot (CT)_n$  on DNA replication has continued. We have confirmed previous results that suggested these sequences slow fork progression by use of a new technique of two dimensional electrophoresis. We have extended previous results to demonstrate that slowing occurs irrespective of sequence orientation.

We are directly testing the role of  $(GA)_n \cdot (CT)_n$  sequences in amplification.

We have concluded an analysis of sequences thought to be enriched for mammalian replication origins and report them to be enriched for (i) a particular transcription regulatory element, (ii) AT-rich sequences (iii) matrix attachment regions and (iv) the ARS consensus sequence for S. cerevisiae but not S. pombe.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01-DK 36101-15 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Energy Conversion in Biology

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Terrell L. Hill	Scientist Emeritus	LMB/NIDDK
Others:	Yi-der Chen	Research Chemist	LMB/NIDDK
	Robert J. Rubin	Special Volunteer	LMB/NIDDK
	R. Blumenthal		LMB/NCI

## COOPERATING UNITS (if any)

J. Lowy, Armed Forces Radiobiology Research Institute, Bethesda, MD  
H. Westerhoff, Netherlands Cancer Institute, Netherlands  
R. Blumenthal, LMB/NCI

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Theoretical Molecular Biology

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.50

## PROFESSIONAL:

1.50

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A number of different topics have been studied in the general field of free energy transduction and biophysics of biological systems. The most important areas in which progress has been made are the study of redistribution of charge carriers near an energized membrane, the time-dependent assembly of microtubule on a bare nucleating site, video microscopic studies of virus-cell fusion, a theoretical study on the use of self-quenching fluorophores in virus-cell fusion kinetics, and the diffusion of lipids or lipid-like molecules between the membranes of a fusing virus-cell complex.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 36102-18 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Statistical Thermodynamics of Protein and Polynucleotide Systems

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Terrell L. Hill Scientist Emeritus LMB/NIDDK

Others: Yi-der Chen Research Chemist LMB/NIDDK

## COOPERATING UNITS (if any)

J. Chalovich, University East Carolina, North Carolina

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Theoretical Molecular Biology

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

.50

## PROFESSIONAL:

.50

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Statistical mechanics were used to derive the binding of a ligand to a one-dimensional lattice in the presence of another ligand which compete with the first ligand. The binding isotherm equations derived for the system was then used to analyze the competitive binding data of myosin subfragment-1 and caldesmon to actin.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 36104-08 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Thermal Measurements of Biomolecular Systems

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: P.D. Ross

Research Chemist

LMB/NIDDK

## OTHERS:

F.B. Howard

Research Chemist

LMB/NIDDK

H.T. Miles

Research Chemist

LMB/NIDDK

## COOPERATING UNITS (if any)

Laboratory of Physical Biology, NIAMS, NIH (A.C. Steven)  
DBBP, CDB, NIH (A. Shrake)

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Section on Physical Chemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2

## PROFESSIONAL:

2

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

1. We have set up and learned to use a commercially available differential scanning calorimeter provided by Dr. A. Steven (NIAMS). With this instrument we have redetermined the thermal melting behavior of the DNA dodecamers containing purine-purine mismatch base pairs (samples provided by F. Howard and H.T. Miles) that were described in last year's report. We have established from the long extrapolations of the pre-transition and post-transition base lines that there is essentially no change in heat capacity in the vicinity of the transition temperature. This hard won and significant result requires revision of our previous interpretations of the DSC data and establishes from a thermodynamic point of view that the thermally induced strand dissociation is a simple two-state process.

2. A computer program has been written (with A. Shrake) for simulation of the excess heat capacity profile of a multi-liganded macromolecule undergoing a reversible thermally induced transformation between native and denatured states. For both the native and denatured forms, provision is made for the introduction of an arbitrary number of ligand binding constants; each with their unique temperature variation. The concentration, molecular weight, transition temperature and the changes in enthalpy and heat capacity accompanying denaturation of the unliganded macromolecule are also entered into the calculation. At each temperature the degree of advancement of the denaturation reaction,  $\alpha$ , is calculated and the multi-ligand equilibria recomputed. The excess heat capacity is obtained from the temperature derivative of  $\alpha$ .

## NOTICE OF INTRAMURAL RESEARCH PROJECT

201 DK 36105-07 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Influences of Macromolecular Crowding on Biochemical Systems

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S.B. Zimmerman Research Chemist LMB, NIDDK

OTHERS: S.O. Trach Research Chemist LMB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Section on Physical Chemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

1.0

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The high concentrations of macromolecules within cells will change the properties of a variety of biochemical reactions through excluded-volume effects. We have considered several methods of correcting parameters determined under conventional dilute solution conditions to conditions more closely approximating the highly volume-occupied conditions within cells. The first method is largely computational: the macromolecular composition within cells is represented as a distribution of hard spherical particles and scaled particle theory is used to calculate the desired activity coefficient for a test sphere of arbitrary size. There are obviously major assumptions made in such a calculation. As an independent test, we are developing an experimental assay for excluded volume in complex mixtures like cell extracts. The assay is based on distribution properties of macromolecules in the phosphate/polyethylene glycol two-phase partition system described by Albertsson. The assay has yielded estimates of excluded volumes in solutions of individual proteins that are consistent with known properties of the proteins. Cell extracts can be manipulated at in vivo concentrations in the assay system. It remains to be seen if the assay is accurate enough with mixtures of macromolecules to estimate excluded volume effects in cell extracts.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 36106-02 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Developmental Regulation of Differential Gene Expression

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: Alan Wolffe, Visiting Associate LMB/NIDDK

## OTHERS:

Constantin Chipev, Visiting Associate LMB/NIDDK

Sherrie Tafuri, IRTA Fellow LMB/NIDDK

## COOPERATING UNITS (if any)

Department of Chemistry, Johns Hopkins University (T. Tullius, J.J. Hayes);  
Division of Biotechnology, CSIRO, Sydney, Australia (H.R. Drew);  
Institut Jacques Monod, Paris, France (M. Mechali, G. Almouzni).

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Section on Physical Chemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.8

## PROFESSIONAL:

1.8

## OTHER:

0

## CHECK APPROPRIATE BOXES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Our work has focussed on the molecular mechanisms responsible for establishing and maintaining stable states of gene expression during vertebrate embryogenesis. Progress has been achieved in three key areas. First, we have demonstrated that interactions between proteins binding to the promoter elements of a gene will have a central role in regulating transcription. Secondly, we have extended our understanding of the role of chromatin structure in preventing transcription factors from associating with genes. This work employs two novel approaches: curved DNA elements have been used to position a nucleosome relative to gene regions of defined function and the hydroxyl radical reagent has been used to provide new information regarding the structure of DNA in a nucleosome. Finally, we have used *in vitro* replication systems derived from *Xenopus* eggs to demonstrate that a competition exists between transcription complex assembly and chromatin assembly on replicating DNA. Moreover we have demonstrated that conformational transitions in chromatin structure can have a dominant effect on differential gene activity. This confirms and extends our earlier demonstration of the specific and dominant repression of genes driven by histone H1 mediated transitions in chromatin structure.

The significance of this work is that it provides direct evidence for an active and central role of chromatin structure in causing the sequestration of the regulatory regions of eukaryotic genes. This role may pervade all regulated processes involving DNA in the eukaryotic nucleus e.g. replication, transcription, recombination, viral latency etc.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 36108-02 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on the Mechanism of Retroviral DNA Integration

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Robert Craigie	Visiting Scientist	LMB/NIDDK
Kiyoshi Mizuuchi	Chief, Section on Genetic Mechanisms	LMB/NIDDK
Martin Gellert	Chief, Section on Metabolic Enzymes	LMB/NIDDK

Others: Tamio Fujiwara	Special Volunteer	LMB/NIDDK
Frederic Bushman	IRTA	LMB/NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Section on Genetic Mechanisms

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

2

## PROFESSIONAL:

2

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Integration of a DNA copy of a retroviral genome into a chromosome of an infected cell is an essential step for normal viral replication. The objectives of this project are to analyze the detailed molecular mechanism of this DNA integration reaction and to develop simple cell-free integration systems that may be used to screen for potential anti-retroviral drugs that block this step of the viral replication cycle.

We have previously demonstrated, with Moloney murine leukemia virus (MoMLV) as a model system, that DNA integration occurs by joining the 3' ends of the viral DNA to the 5' ends of a staggered cut made in the target DNA. Subsequently, we developed an *in vitro* system that accomplishes this reaction. The DNA substrate for integration (mini-MoMLV) is a linear plasmid with ends that mimic the ends of authentic unintegrated MoMLV DNA. The viral proteins necessary for integration are provided as disrupted MoMLV virions. Inclusion of a cytoplasmic extract of uninfected cells in the reaction mixture greatly enhances the efficiency of integration. With this cell-free integration system as the assay, we have determined that the MoMLV IN protein is the only viral protein that is essential for integration. This protein has been cloned and expressed using the Baculovirus expression system, and has been purified to greater than 90% homogeneity. The cloned MoMLV IN protein substitutes for disrupted MoMLV virions as the source of viral protein in our *in vitro* integration reaction.

The detailed mechanistic information we have obtained concerning the MoMLV DNA integration reaction is now being applied to develop a parallel *in vitro* system for integration of HIV. We have cloned and expressed the HIV IN protein, using the same methodology as for MoMLV. Integration of mini-HIV DNA mediated by the cloned HIV IN protein has been detected at low frequency in preliminary experiments.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 36109-02LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

AIDS related proteins: Structure and function

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: David R. Davies, Chief, Sect. on Molecular Structure LMB/NIDDK

Others: Enid W. Silverton, Research Chemist LMB/NIDDK  
Eduardo A. Padlan, Visiting Scientist LMB/NIDDK  
Arthur B. Shaw, Expert LMB/NIDDK  
Christina Brown, Visiting Fellow LMB/NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Section on Molecular Structure

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.25

## PROFESSIONAL:

1.25

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

1) A potential inhibitor of the HIV protease has been synthesized, and tested against the HIV protease. Tests showed less than micromolar affinity.

2) We have attempted to crystallize the CD4 antigen complexed to the Fab of a monoclonal antibody to CD4 that blocks the binding of GP120. So far, the Fab OKT4a has been crystallized. Preliminary X-ray analysis shows that this is a promising candidate for further structure determination.

3) We have attempted to capitalize several other proteins from the HIV. These include REV and NEF.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 36110-01 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Control of Gene Expression during Chicken Erythrocyte Development

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Joanne Nickol, Research Chemist LMB/NIDDK

## OTHERS:

Zhen-yong Zhang-Keck, Special Volunteer LMB/NIDDK

## COOPERATING UNITS (if any)

Salk Institute (B. Emerson)

Lab. of Molecular Carcinogenesis, NICK, NIH (M. Crippa, M. Bustin)

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Section on Physical Chemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

.75

## PROFESSIONAL:

.75

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

Developmental regulation of expression of two major gene families were studied: globin genes and HMG 14(a and b) and 17 genes. The former represents a differentiated function restricted to erythrocytes whereas the latter genes are constitutively expressed as an essential component of cellular chromatin.

A. Globin genes. Expression of the  $\epsilon$  globin gene is restricted to the primitive erythrocyte, which circulates only early in chick embryogenesis (day 1-7). The promoter has been sequenced and putative control regions are being analyzed by in vitro footprinting and gel mobility shift assays. The extracts used for these analyses are derived from erythrocytes obtained at different stages of development spanning the time when the  $\epsilon$  gene is fully active to the adult chicken, when all genes are totally inactive. These regions of differential binding within the  $\epsilon$  promoter are also being analyzed for functional activity by linkage to a reporter gene and subsequent transfection into primary erythrocytes.

B. HMG 14(a and b) and 17. To establish this system we investigated various parameters related to expression of these genes in erythrocytes during embryogenesis: pulse-labeled protein, steady state mRNA, nuclear run-ons that analyze only newly synthesized transcripts, and chromatin structure of the genes themselves. Our results suggest that the individual genes of the HMG family are differentially expressed in development. HMG 14a, in particular, is restricted to the primitive (early) erythrocyte whereas HMG 14b and 17 are maximally expressed only later, in the definitive erythrocyte, but not in the reticulocyte.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 36111-01 LMB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structural Molecular Biology

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Joanne Nickol, Research Chemist LMB/NIDDK

## COOPERATING UNITS (if any)

Laboratory of Biochemical Metabolism, NIDDK, NIH (D. Rau);  
University of Calgary Medical School, Calgary, Alberta, Canada (D. Bazett  
Jones, M. Brown).

## LAB/BRANCH

Laboratory of Molecular Biology

## SECTION

Section on Physical Chemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

.25

## PROFESSIONAL:

.25

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Alternative non-B DNA structures may play a role in gene function by modulating interactions with regulatory proteins. We are using two related physical techniques to analyze possible conformations of DNA and DNA-protein complexes.

A. Birefringence. We have analyzed the persistence length of a 250bp fragment of DNA containing the 5S RNA gene of *Xenopus borealis*. In the presence of Zinc (+2) or spermidine this fragment has an altered structure not assumed by a control piece of DNA. We are currently analyzing this 5S RNA gene complexed to TFIIIA, a protein which binds in the middle of the gene and is essential for transcription.

B. Photochemical Electric Dichroism. We have developed a new technique for analyzing DNA structure and DNA-protein interaction. It involves combination of photochemical techniques (generation of UV pyrimidine dimer crosslinks by laser illumination) with electric dichroism (orientation of DNA in a high electric field). We have analyzed the 5S RNA gene of sea urchins and have found an alternate DNA structure associated with oligopurine tracts within the TFIIIA-binding domain of this gene.



ANNUAL REPORT OF THE METABOLIC DISEASES BRANCH  
National Institute of Diabetes and Digestive and Kidney Diseases

The general goals of the Branch are to investigate the mechanism of action of hormones controlling ion transport and mineral metabolism and to investigate the immunological and pathological factors mediating kidney disorders. The branch currently includes sections of Mineral Metabolism (Dr. Marx), Endocrine Regulation (Dr. Aurbach) and Kidney Disease (Dr. Balow). Integration of these sections is related to common interests in the pathophysiology of metabolic disorders which interface with the kidney. Systems under study include renal and skeletal tissue, transgenic mice, isolated cells (kidney and parathyroid in culture, hormone receptors (beta adrenergic, parathyroid hormone, calcitonin and 1,25 dihydroxy vitamin D), parathyroid cell growth factors, and T cell and B cell function in disorders of immunoregulation.

#### Analysis of Hormone Receptor

Interactions with several hormone receptors regulating growth or adenylate cyclase are under study. Specific receptors have now been identified on turkey erythrocytes, parathyroid cells, pineal cells, rat, guinea pig and monkey lung membrane preparations, rat osteosarcoma cells and rat liver membranes. Control of receptor in isolated cell culture systems is being studied with a view toward gaining knowledge about the molecular biology of receptors and how they are linked to intracellular response systems.

Calcitonin has been shown to decrease intracellular cAMP at concentrations 300-fold lower than those that increase cAMP [Drs. Barsony, Marx].

#### Primary Hyperparathyroidism and Familial Hypercalcemia

Clinical studies are continuing on primary hyperparathyroidism and its familial variants. Detailed family screening and case findings have produced approximately 85 kindreds for analysis. These studies allowed segregation of the most common familial variants into two apparently distinct disease syndromes - familial multiple endocrine neoplasia type 1 (FMEN I) and familial hypocalciuric hypercalcemia (FHH). FHH was distinguished from FMEN I by 1) virtually a 100% penetrance for hypercalcemia before age 20, 2) milder clinical manifestations - low incidence of recurrent nephrolithiasis or recurrent peptic ulceration, 3) no hypercalciuria, 4) normal basal concentrations of gastrin, and 5) poor response to subtotal parathyroidectomy.

Distinction between the two syndromes, both inherited as autosomal dominant traits, is important because in FHH the clinical course is generally milder and subtotal parathyroidectomy is less likely to be beneficial. FHH accounts for approximately 10% of all unsuccessful parathyroidectomies in hypercalcemia. In FHH the ionized and ultrafiltrable calcium concentration in serum are elevated in proportion to the increase in total calcium. In these patients the filtrable load of calcium is high in association with a marked decrease in renal calcium clearance. Even when these patients become surgically hypoparathyroid, the low renal clearance of calcium is strikingly persistent during calcium infusion. The concentration of parathyroid hormone in plasma is lower in patients with FHH than in typical primary hyperparathyroid patients with similar degrees of hypercalcemia whether assessed by PTH radioimmunoassay or by renal clearance of cAMP or phosphate. The parathyroid glands show hyperplasia in most cases. In several kindreds one or more members have exhibited life-threatening primary hyperparathyroidism in the neonatal period. This may result sometimes from a double dose of the FHH gene. Dispersed parathyroid cells from one severely affected neonate showed a striking decrease in sensitivity of PTH secretion to extracellular calcium. This disorder may reflect mutation in a gene that directs calcium recognition in both the parathyroid and renal tubular cell [Drs. Marx, Streten, Zimering and Aurbach. MDB; Drs. Spiegel and Weinstein MPB].

Familial multiple endocrine neoplasia type I (FMENI) is an autosomal dominant disorder characterized by hyperfunction of parathyroids, pancreatic islets, and anterior pituitary. Affected organs show features suggestive of increased proliferation. Virtually all subjects expressing the gene show primary hyperparathyroidism. Primary hyperparathyroidism is usually first recognizable between ages 20-40, and it shows a high recurrence rate after subtotal parathyroidectomy (approximately 50% after 10 years). We have evaluated multiple indices for use in screening in a very large kindred. We tested 221 members and newly identified 16 as carriers. Albumin-adjusted calcium and PTH were most useful; gastrin and prolactin analyses were not useful for screening but showed promise in followup of known carriers. Analysis in this family has revealed linkage to a locus on the long arm of chromosome 11. MENI related tumors are being screened for loss of heterozygosity at this locus. Tumors with small deletions could speed identification of the MENI gene. [Drs. Marx, S. Bale, A. Bale, Mulvihill, Sparkes, Brandi, Aurbach, Sakaguchi].

With cultured bovine parathyroid cells, we found abnormally high mitogenic activity in plasma from 23 of 27 subjects with FMENI. Well-characterized growth factors or known parathyroid secretagogues showed far less parathyroid mitogenic activity than these FMENI plasmas. The mitogenic factor(s) appear to be a protein of 14,000 mw. We have begun purifying this factor for further characterization. We have obtained evidence that the

factor is related to basic fibroblast growth factor. Analysis of plasmas from one large kindred with FMEN1 suggests that high parathyroid mitogenic activity precedes primary hyperparathyroidism and may begin at very early ages. [Drs. Zimering Brandi, Sakaguchi, Aurbach, Goldsmith, Marx].

Studies on noninvasive and invasive modes of localizing parathyroid tumors continue. Parathyroid adenoma localization has been evaluated using the new non-invasive magnetic resonance imaging technique. Initial results were disappointing but the acquisition of a specialized neck collar has led to better resolution in the paratracheal and mediastinal areas. Patients are currently under evaluation with this new technique. A high degree of success has been obtained in localizing tumors through vascular catheterization procedures. Parathyroid arteriography developed and performed by Dr. John Doppman afforded, in approximately 450 of cases tested, the identification of abnormal masses of tissue proven at surgery to be parathyroid. In the most difficult cases, localization of parathyroid tissue can be aided by identifying high concentrations of parathyroid hormone by radioimmunoassay in veins draining the lesion. Fine needle aspiration is another new method that can obviate other invasive localization procedures. We have aspirated with guidance by computerized tomography or ultrasound approximately 20 such lesions that were subsequently confirmed surgically as parathyroid. RIA of the aspirates showed high concentrations of PTH in all but one. Eight mediastinal adenomas have been treated nonsurgically by percutaneous injection via catheter of occlusive agents into the arterial blood supply with 7 complete and one partial remissions. [Drs. Aurbach, Marx, Spiegel, Nanes, Zimering, Weinstein, Streeten, NIDDK: Dr. Norton, NCI, Drs. Doppman, Miller, and others, Diagnostic Radiology, CC].

Rapid determination of intraoperative UcAMP excretion (using the Gammaflo machine for rapid cAMP radioimmunoassay) has proven to be a valuable tool in guiding surgery for primary hyperparathyroidism, particularly in patients with multigland disease. Persistent elevation of UcAMP requires continued search for abnormal tissue even after 1 or more abnormal glands have been removed. A rapid (mean 1.5 hours) drop in UcAMP to less than 50% of the baseline obviates the need for continued exploration even in cases where histologic confirmation of parathyroidectomy is lacking. Spurts in UcAMP above baseline may provide a clue to the location of abnormal parathyroid tissue. [Drs. Spiegel, Marx, Nanes, Zimering, Weinstein, Streeten, and Aurbach, NIDDK: Dr. Norton, NCI Surgery].

Determination of urinary cAMP excretion postoperatively in patients undergoing neck exploration for primary hyperparathyroidism is a useful method for assessing postoperative parathyroid function. UcAMP excretion declines postoperatively in all patients in whom hypercalcemia is corrected but not in those with persistent hypercalcemia. In patients becoming severely hypocalcemic (and requiring vitamin D

therapy) postoperatively, UcAMP measurement enables one to distinguish patients with decreased parathyroid reserve as the cause for hypocalcemia (low UcAMP excretion) from patients with healing osteitis fibrosa ("hungry bones") with secondary hyperparathyroidism as the basis for hypocalcemia. UcAMP in the latter group is often elevated but can be suppressed if serum calcium is normalized. Elevated UcAMP excretion postoperatively in the face of hypocalcemia enables one to predict that vitamin D therapy will be required temporarily (if at all) and precludes the need for parathyroid autografts. [Drs. Spiegel, Marx, Zimring, Weinstein, Streeten, and Aurbach, NIDDK].

Postoperative patients with surgically corrected hyperparathyroidism are being actively evaluated in a five year follow up study [Dr. Udelsman, Norton NCI, Drs. Marx, NIDDK]. These patients are being studied for sequelae such as hypoparathyroidism, recurrent hyperparathyroidism, and complications such as vocal cord paralysis.

#### Secretion of Parathyroid Hormone

PTH secretion from parathyroid glands in vivo and cells in vitro is controlled by intracellular calcium and cyclic AMP. Control by calcium is altered in certain pathologic states (glandular adenomas, carcinomas and perhaps hyperplasia). Agents that alter cellular cAMP change PTH secretion in the same direction. Calcium decreases cellular cAMP, but most of its effect to inhibit secretion is independent of changes in cellular cAMP.

Calcium inhibition of parathyroid hormone secretion was evaluated utilizing pertussis toxin as a probe. Pertussis toxin catalyzes ADP- ribosylation and inactivation of inhibitory guanine nucleotide regulatory proteins,  $N_i$ 's. Studies in dispersed bovine parathyroid cells indicate that calcium inhibition of parathyroid hormone secretion is mediated via an  $N_i$ . Further studies with calcium channel agents show that calcium channels are involved in regulation of PTH secretion. Two classes of antibodies against a calcium channel protein influence parathyroid secretion. One, an agonist inhibits secretion (by opening the calcium channel). The other, an antagonist, promotes secretion. [Drs. Fitzpatrick, Chin, Nirenberg, and Aurbach].

A cloned rat parathyroid cell line (PT-r) has been developed. Proteoglycans synthesized by the PT-r cell have been characterized and effects of calcium on synthesis studied. One proteoglycan is located both intracellularly and on the external surface of the cell. Distribution of this proteoglycan between cell cytosol and cell surface is controlled by calcium. This effect on cell surface proteoglycans may be important in calcium regulation of growth as well as secretion. [Drs. Yanagishita, Sakaguchi, Brandi, and Aurbach].

We have also cloned endothelial cells from bovine parathyroid tissue. These cells are distinct from the parathyroid epithelial cells that secrete hormone. As is characteristic of endothelial cells they contain Factor VIII-related antigen, take up acetylated low density lipoprotein and show ultrastructural features comparable to other endothelial cells. Using this cloned cell system we have shown that it is the target of antibodies developed in autoimmune hypoparathyroidism and also is the target for a parathyroid cell growth factor identified in multiple endocrine neoplasia type I. [Drs. Brandi, Sakaguchi, Zimering, Falchetti, and Aurbach].

#### Vitamin D Resistance and Related Disorders

The role of  $1,25(\text{OH})_2\text{D}_3$ , the most potent natural metabolite of vitamin D, has been assessed in hypocalcemic states. This very rapidly acting drug has simplified the management of hypocalcemia following parathyroidectomy: during this time skeletal remineralization imposes large but rapidly diminishing requirements for calcium.

We have evaluated patients with extreme resistance to  $1,25(\text{OH})_2\text{D}$ . This can be a transient state as following parathyroidectomy or a permanent state as in familial cases. We have evaluated 20 patients with familial resistance to  $1,25(\text{OH})_2\text{D}$ . Most patients have hypocalcemic rickets, usually with associated total alopecia. The alopecia is associated with the highest grades of resistance to  $1,25(\text{OH})_2\text{D}$ , implicating calcitriol in physiology of the hair follicle. Mineral homeostasis is usually improved by treatments that sustain  $1,25(\text{OH})_2\text{D}$  levels at 10-100 times normal. One patient had absent intestinal response to  $1,25(\text{OH})_2\text{D}$ , documented with a new stable isotope technique [Drs. Yergey, Viera, Bliziotis, Nanes, Marx]. Treatment with high doses of calcium intravenously each day for 4 months caused dramatic clinical improvement, showing that calcium could replace most functions of the  $1,25(\text{OH})_2\text{D}$  receptor.

Specific intracellular defects have been evaluated using cultured skin fibroblasts from these patients. With skin fibroblasts cultured from normals, a typical  $1,25(\text{OH})_2\text{D}$ -receptor can be identified by binding in soluble extracts, by nuclear uptake of hormone with intact cells, or by elution of occupied receptor from DNA-cellulose. Fibroblasts from patients with familial resistance to  $1,25(\text{OH})_2$  have shown a spectrum of defects including nonfunctional receptors, diminished numbers of receptors, and receptors with decreased hormone binding affinity. Among cases with normal hormone binding sites on the receptors some show receptors with deficient binding to nucleus while others show normal binding to nucleus but abnormal interaction with nonspecific DNA (as DNA-cellulose). In one patient, osteoblast-like cells from bone biopsy exhibited a defect analogous to that in skin fibroblasts of the same patient. Even when receptors have unmeasurable hormone-binding activity, the

receptor protein has been present in normal amounts according to immunoassay suggesting small mutations in the hormone-binding region. Cellular action of  $1,25(\text{OH})_2\text{D}_3$  can be analyzed by measuring its induction of the  $25(\text{OH})\text{D}$  24-hydroxylase enzyme system. Cultured skin fibroblasts from all patients with hereditary resistance to  $1,25(\text{OH})_2\text{D}$  exhibit defects in this induction. [Drs. Marx, Barsony, Brandi, Nanes, MDB, NIDDK; Dr. Liberman, Israel; Drs. Pike and Haussler, U. Arizona].

New world primates show resistance to many steroid hormones, including  $1,25(\text{OH})_2\text{D}$ . EB virus transformed B lymphocytes from a new world primate showed receptors with lower affinity and capacity for  $1,25(\text{OH})_2\text{D}_3$  than in similar cells from old world primates (human or macaque) [Drs. Marx; Liberman, (Israel)].

Fibroblast lines from patients with hereditary extreme resistance to  $1,25(\text{OH})_2\text{D}_3$  are being used to probe for normal functions of the  $1,25(\text{OH})_2\text{D}_3$  receptor. We have shown that  $1,25(\text{OH})_2\text{D}_3$  can elevate intracellular cyclic GMP very rapidly (within 1-3 minutes). This response showed affinity and analog specificity characteristic of the  $1,25(\text{OH})_2\text{D}_3$  receptor and was absent in all "mutant" fibroblast lines although they retained a rapid cGMP response to nitroprusside and to androgens. Thus a  $1,25(\text{OH})_2\text{D}_3$  receptor mediates this rapid response. [Drs. Barsony, Marx].

## KIDNEY DISEASES

The Kidney Disease Section conducts research focussed on the clinical and pathologic features of immunologically mediated glomerular diseases. Specific entities include the proliferative and membranous forms of lupus nephritis, idiopathic membranous nephropathy and membranoproliferative glomerulonephritis. Human subjects, as well as animal models, are intensively studied to develop insights into pathogenetic mechanisms and to test novel immunosuppressive drug therapies which might have salutary effects on the course of these nephropathies.

### I. Glomerulonephritis and lupus nephritis

A. Immunopathogenesis. Murine models are being utilized to investigate the different forms and components of lupus nephritis. Characteristics of the immune complex deposits and lymphoid cell localization in the kidney are being studied by immunohistologic and electron microscopic techniques. The modulating effects of cyclophosphamide on immune responses and on the renal lesions are being investigated. Studies of differences among the murine strains have provided new approaches to study of the diverse manifestations and response to treatment of human lupus nephritis. (Austin, Cadena, Balow).

B. Immunoregulatory studies. Heightened and poorly regulated B lymphocyte activity is characteristic of systemic lupus erythematosus (SLE). Defective T suppressor cell activity is an inconsistent finding in SLE and does not fully explain excessive B lymphocyte responses. Moreover, T cytotoxic cell and natural killer cell activities are deficient and could permit the emergence of abnormal and unregulated autoantibody producing cells. An alternative immunoregulatory defect leading to excessive B lymphocyte activity has been noted in certain lupus mouse strains, namely, T helper lymphocyte hyperactivity. Our group has found increased numbers of circulating T cells bearing activation markers and proto-oncogene expression which may lead to increased immunoglobulin secretion by autologous B cells. Studies are in progress to ascertain whether different mechanisms are responsible for the heightened production of pathogenic antibodies by B lymphocytes in different subsets of patients with lupus nephritis. (Tsokos, Boumpas, Yamada, Patel, Balow).

C. Proliferative lupus nephritis. Current protocols are designed to increase and refine the therapeutic index of different immunosuppressive drugs for lupus nephritis. Studies to date have shown that cytotoxic drugs are superior to corticosteroid therapy and that intermittent cyclophosphamide therapy maintains efficacy while reducing toxicity. Patients with proliferative forms of lupus nephritis are being intensively treated with pulse methylprednisolone or pulse cyclophosphamide to compare these two types of drugs and to assess whether intensity or duration of cyclophosphamide therapy is more important in stabilizing the renal disease. Laboratory studies of lymphoid cell modulation by the

various drug regimens are ongoing in order to identify techniques which will maximize efficacy and to improve monitoring of drug treatment. (Balow, Austin, Webb and members of ARB, NIAMS).

D. Membranous nephropathy. Membranous nephropathy produces substantial morbidity from nephrotic syndrome and causes an insidious loss of renal function in patients with lupus and in those patients with idiopathic forms of this disease. Preliminary evidence indicates that the immunopathogenesis of membranous nephropathy is distinct from that of most proliferative forms of glomerulonephritis. Current protocols involve examination of the pathophysiology and histopathology of the glomerular lesions in membranous nephropathy, as well as evaluation of the comparative efficacy of prednisone, cyclophosphamide and cyclosporin A in patients idiopathic and lupus related forms of this renal disease. (Balow, Austin, Webb).

## II. Role of Complement in Glomerulonephritis.

A. Nephritic factors. Patients with membranoproliferative glomerulonephritis and lupus nephritis develop autoantibodies reactive with complement converting enzymes which leads to abnormal consumption of complement components. These nephritic factors may participate in the pathogenesis of the renal diseases, but studies of their exact role has been hindered by insufficient quantities of homogeneous materials. Epstein-Barr virus transformed and sustained B lymphocyte lines which actively produce nephritic factors have been produced. One line from a patient with membranoproliferative glomerulonephritis secretes an IgG antibody which binds and stabilizes the alternative pathway C3 convertase enzyme. Another from a patient with lupus binds the classic pathway C3 convertase. Anti-idiotypic antibodies to nephritic factors have been isolated. The characteristics of ligand binding, receptor turnover and modulation by the nephritic factors, and idiotypic binding sites are under study. (Tsokos, Thyphronitis, Anastassiou, Balow).

B. Complement in immune regulation. Abnormal levels of complement components and deposition in sites of immunological reactions are characteristic of several forms of nephritis. The interactions of complement components and activation products with receptors on lymphoid cells are being studied to gain new insights into their potential role in lupus nephritis, membranoproliferative glomerulonephritis and other renal disorders. A deficiency in number or function of complement receptors on B lymphocyte may predispose to the appearance of autoantibodies associated with these diseases. Studies are underway to determine the mechanism of the modulation of B lymphocyte responses through interaction of the complement receptor with natural complement ligands, Epstein-Barr virus, monoclonal antibodies and anti-IgM with which complement receptors co-cap under certain conditions of B lymphocyte activation. (Tsokos, Thyphronitis, Anastassiou, Balow).

## Studies of the pathogenesis of glomerulosclerosis

The renal cell biology unit is interested in the cellular mechanisms leading to glomerular scarring, the emphasis being on non-immunologically mediated diseases. The general hypothesis is that abnormalities in the growth regulation of resident glomerular cells play a major role in the development of glomerulosclerosis. We have developed methods using both in vitro approaches with glomerular cell culture and in vivo techniques using transgenic mice and the newly developed nonobese diabetic mouse (NOD). (L. Striker, G. Striker, T. Doi, K. MacKay, F. Conti, S. Elliot, R. Perfetti and L. Agodoa)

### III. Glomerulosclerosis

#### A. In Vivo Studies.

1. SV40 transgenic mice: Mice transgenic for the early region of simian virus 40 develop proteinuria and progressive glomerulosclerosis. We have postulated that large T antigen causes abnormal glomerular cell proliferation which induces glomerulosclerosis after unilateral nephrectomy. We found that unilateral nephrectomy markedly increased the severity of the glomerulosclerotic lesions in female mice but not in males. The sclerosis was associated with a marked increase in the size of the glomerular profiles. (K. MacKay, L. Striker, G. Striker)

2. Mice transgenic for GH, GHRF and IGF-I: We found that mice transgenic for GH and GHRF develop severe progressive glomerulosclerosis with an increase in the size of the glomeruli. By contrast, in IGF-I transgenic mice, while the glomeruli are also moderately enlarged, they do not develop glomerulosclerosis. This suggests that overexpression of certain growth factors, such as growth hormone and growth hormone releasing factor, may have effects in addition to those on cell growth. These may be important in inducing those additional changes which result in glomerulosclerosis. The elucidation of the pathogenetic events in this model may lead to further understanding of the general pathogenesis of glomerulosclerotic lesions. (T. Doi, L. Striker, G. Striker)

3. Nonobese diabetic (NOD) mice: NOD mice develop autoimmune diabetes mellitus, which predominates in females and leads to death if untreated with insulin. We compared the glomerular size, morphology, composition of the glomerulosclerosis and urinary abnormalities in mice with and without overt diabetes. Hyperglycemia was rapidly followed by an increase in glomerular size, mesangial sclerosis and proteinuria. Collagen deposition was markedly increased in the mesangial areas. These mice could provide a good model of nephropathy in a

genetically determined model of diabetes mellitus. This study suggests that hyperglycemia triggers rapid glomerular lesions, if the glomeruli have a genetic propensity to develop sclerosis. (T. Doi, L. Striker, G. Striker)

4. Kidney disease in diabetic Pima Indians. We have undertaken a retrospective study of the glomerular lesions occurring in diabetic Pima Indians. They have a very high frequency of NIDDM. We have developed morphometric methods to measure the glomerular surfaces and determine whether glomerulosclerosis is associated with hypertrophy. (L. Striker, P. Bennett, G. Striker)

#### B. In Vitro Studies.

1. Murine and human glomerular cells: We have developed lines of mouse epithelial, mesangial and endothelial cells from normal mice and have been investigating their response to growth peptides (IGF-I, TGF-beta) and insulin. We have also undertaken studies on the role of IGF-I in the cell cycle of human mesangial cells, and provided evidence that it is a progression factor. Finally, we have shown that mouse mesangial cells not only respond to, but also produce, IGF-I immunoreactive molecules and IGFs binding proteins. This suggests that these peptides may play an important role in glomerular function. (T. Doi, S. Elliot, L. Striker, G. Striker)

2. Transforming growth factor-beta: We have explored the regulatory effects of TGF-beta on murine glomerular cells. Mouse epithelial, mesangial and endothelial cells as well as isolated rat glomeruli possess high affinity receptors for TGF-beta. While TGF-beta inhibits the proliferation of epithelial and endothelial cells, it acts as a bifunctional regulator of mesangial cell proliferation. TGF-beta increases the biosynthesis of fibronectin and collagen by mesangial cells. These data suggest that TGF-beta may play an important role in vivo, in diseases characterized by glomerular cell proliferation and accumulation of extracellular matrix. (K. MacKay, L. Striker, G. Striker)

3. Endothelial cells and insulin: We have identified a receptor for insulin on the surface of a clone of endothelial cells from normal mice. (S. Elliot, F. Conti, L. Striker, G. Striker)

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43002-24 MDB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structure, Secretion and Mechanism of Action of Parathyroid Hormone

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: G.D. Aurbach, M.D.

Chief, MDB, NIDDK

OTHERS: L.A. Fitzpatrick, M.D.

Former Senior Staff Fellow, MDB, NIDDK

M.L. Brandi, M.D., Ph.D.

Visiting Associate, MDB, NIDDK

K. Sakaguchi, M.D., Ph.D.

Visiting Associate, MDB, NIDDK

M. Zimering, M.D.

Medical Staff Fellow, MDB, NIDDK

Y. Fujii, M.D.

Visiting Fellow, MDB, NIDDK

A. Falchetti, M.D.

Former Guest Worker, MDB, NIDDK

COOPERATING UNITS (If any) Bone Research Branch, NIDR

Laboratory of Biochemical Genetics, NHLI

Endocrine Unit, Massachusetts General Hospital

Department of Medicine Yale University

## LAB/BRANCH

Metabolic Diseases Branch

## SECTION

Endocrine Regulation Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4

## PROFESSIONAL:

2.25

## OTHER:

1.75

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

It is the purpose of this project to study the secretion, function, and mechanism of action of parathyroid hormone, its relationship to human disease, and to develop clinically useful tests for circulating parathyroid hormone. From these studies it is expected that one can understand the pathophysiology of certain metabolic diseases of bone and endocrine disturbances. The entire structures of bovine, porcine, rat and human parathyroid hormone have been determined. Synthetic polypeptides representing bovine rat and human parathyroid hormone have been synthesized. These molecules show all the biological properties of the native hormonal polypeptides. Highly sensitive radioimmunoassays for the hormone have been developed and are being modified further for improved clinical diagnostic parameters. Studies show that the mechanism of action of the hormone is mediated through direct hormonal activation of adenylate cyclase in bone and kidney. Isolated parathyroid cells and culture systems have been developed that allow studies on secretory control of parathyroid hormone, and provide test systems to elucidate the pathophysiology of certain hypoparathyroid and hyperparathyroid states.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43003-24 MDB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on the Mode of Action of Thyrocalcitonin

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S.J. Marx, M.D. Chief, Min. Metab. Sec. MDB, NIDDK

Others: J. Barsony, M.D. Visiting Associate MDB, NIDDK

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Metabolic Diseases Branch

## SECTION

Mineral Metabolism Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

0.1

## PROFESSIONAL:

0.1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose is to study the interaction of calcitonin with its specific receptor target organs. The current investigations should provide further insight into the structure-function relationship in calcitonin. Calcitonin is a small polypeptide hormone and therefore lends itself well to studies using synthetic peptide fragments. The system is also useful for characterizing hormone receptors in kidney, bone and other tissues. Studies are in progress to characterize further the interaction of calcitonin with tissue receptors. It will also be of interest to solubilize the receptors and characterize them chemically. Calcitonin increases cAMP in MCF 7 breast cancer cells. At 300-fold lower concentration calcitonin decreases cAMP in these cells. The decrease in cAMP is prevented by preexposure of cells to agents that interfere with inhibitory guanyl regulatory proteins.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43006-14 MDB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Study of Hyperparathyroidism: Etiology, Diagnosis and Treatment

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: G.D. Aurbach, M.D.

Chief, MDB, NIDDK

OTHERS: S.J. Marx, M.D.

Chief, Min. Met. Sec., MDB, NIDDK

E. Friedman, M.D.

Medical Staff Fellow, MPB, NIDDK

J. Merendino, M.D.

Medical Staff Fellow, MPB, NIDDK

M.L. Brandi, M.D., Ph.D.

Visiting Associate, MDB, NIDDK

K. Sakaguchi, M.D., Ph.D.

Visiting Associate, MDB, NIDDK

M. Zimering, M.D.

Medical Staff Fellow, MDB, NIDDK

E. Streeten, M.D.

Medical Staff Fellow, MDB, NIDDK

## COOPERATING UNITS (if any)

Radiology Department, CC; Surgery Branch, NCI; Digestive Diseases Branch, NIDDK

## LAB/BRANCH

Metabolic Diseases Branch

## SECTION

Endocrine Regulation Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

4.75

## PROFESSIONAL:

2.50

## OTHER:

2.25

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☒ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The project goal is the evaluation and treatment of hyperparathyroidism. Patients with persistent or recurrent hyperparathyroidism are referred for evaluation and treatment. Hereditary hyperparathyroidism in particular is under investigation in the hopes of delineating hereditary molecular abnormalities in glandular regulation, as exemplified in the multiple endocrine neoplasia syndromes. Evaluation ranges from epidemiologic studies of families to in-house clinical studies of patients and to in vitro analyses of excised tissue. Techniques currently being employed and improved include radioimmunoassay of parathyroid hormone, ultrasonography, radiothallium scanning, magnetic resonance imaging, CAT scanning, selective arteriography and selective venous sampling for parathyroid hormone, parathyroid gland cryopreservation and autotransplantation, and transcatheter parathyroid gland infarction. In vitro evaluation of parathyroid and other endocrine tissue involves tissue culture, chemistry and determination of linkage with DNA or RNA probes.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43008-08 MDB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Vitamin D Resistance and Related Disorders

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	S.J. Marx, M.D.	Chief, Min. Metab. Sec.	MDB, NIDDK
Others:	J. Barsony, M.D.	Visiting Associate	MDB, NIDDK
	W. McKoy	Chemist	MDB, NIDDK
	L. De Marco, M.D., Ph.D.	Special Volunteer	MDB, NIDDK
	C. Smith, Ph.D.	IRTA	MDB, NIDDK

## COOPERATING UNITS (if any)

Metabolism Unit, Beilinson Hospital, Petah Tiva, Israel	(U. Liberman)
Cell Biology Department, Baylor University	(J.W. Pike)
Biochemistry Department, University of Wisconsin, Madison	(H.F. DeLuca)
Hormone Action and Oncogenesis Section, NCI	(G. Hager)

## LAB/BRANCH

Metabolic Diseases Branch

## SECTION

Mineral Metabolism Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

3

## PROFESSIONAL:

2.5

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The calciferols were the first class of hormonally active steroids to be discovered and also the first for which subjects with hormone resistance could be identified. With recognition that vitamin D is the precursor for 1,25-dihydroxyvitamin D, it has become possible to characterize defects in the activation (1-hydroxylation) of vitamin D and defects in the target action of activated (1,25-dihydroxy)vitamin D. We have demonstrated a broad spectrum of manifestations of hereditary resistance to 1,25(OH)<sub>2</sub>D ranging from infantile rickets with alopecia and no intestinal response to calciferols to adult onset osteomalacia with satisfactory intestinal response to high doses of calciferols and with no epidermal abnormalities. Alopecia is found only in cases with the most severe grades of resistance to 1,25(OH)<sub>2</sub>D. This finding implicates the 1,25(OH)<sub>2</sub>D receptor, for the first time, in normal function of a tissue (hair follicle) outside the classical target in duodenal mucosa. Cultured skin fibroblasts display many components of the 1,25(OH)<sub>2</sub>D effector system. Skin fibroblasts from all subjects with hereditary resistance to 1,25(OH)<sub>2</sub>D display abnormalities in this effector system, and defects in many discrete steps of this pathway have been identified with these cells. Cells with mutations in the 1,25(OH)<sub>2</sub>D effector pathway can be used to explore mechanisms of calciferol action. They have been used to establish that the 1,25(OH)<sub>2</sub>D receptor mediates an extremely rapid (1-3 minutes) rise of cyclic GMP in response to 1,25(OH)<sub>2</sub>D<sub>3</sub> and that certain receptor mutations compromise many receptor functions but allow another function to be retained normally. This establishes that 1,25(OH)<sub>2</sub>D receptors couple to different responses by distinct mechanisms.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43009-04 MDB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Mineral Metabolism

PI: S.J. Marx, M.D.

Chief, Min. Metab. Sec.

MDB, NIDDK

## Others:

M.L. Brandi, M.D.

Visiting Associate

MDB, NIDDK

W. McKoy

Chemist

MDB, NIDDK

G. Aurbach, M.D.

Chief

MDB, NIDDK

E. Streeten, M.D.

Medical Staff Fellow

MDB, NIDDK

K. Sakaguchi, M.D.

Visiting Associate

MDB, NIDDK

COOPERATING UNITS (if any) MPB, NIDDK (A. Spiegel and E. Friedman); EEB, NCI (S. Bale); CEB, NCI (J. Mulvihill); SB, NCI (J. Norton); Belvedere Medical Center, Carlisle, PA; Genetics Department, Yale University (A. Bale)

## LAB/BRANCH

Metabolic Diseases Branch

## SECTION

Mineral Metabolism Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.0

## PROFESSIONAL:

1.5

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects☐ (b) Human tissues☐ (c) Neither☒ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Disorders of mineral metabolism have been evaluated with methods extending from epidemiology to cellular and molecular biology. Two forms of familial hyperparathyroidism have been characterized in detail. Familial hypocalciuric hypercalcemia is an autosomal dominant trait associated with abnormal interactions with calcium in parathyroid and kidney. Familial multiple endocrine neoplasia type 1 (FMEN1) is an autosomal dominant trait causing hyperfunction of parathyroids, pancreatic islet and anterior pituitary. It is associated with gradual but abnormal proliferation of the tissues affected. Genetic linkage studies in a large kindred have localized the MEN1 gene to the long arm of chromosome 11. Plasma from affected persons shows high mitogenic activity upon cultured bovine parathyroid cells. This mitogenic activity in plasma may contribute to primary hyperparathyroidism in FMEN1. Analysis of blood and parathyroid tumor DNA has revealed that FMEN1 parathyroids often show clonal loss of alleles in the region of the FMEN1 gene on chromosome 11. Thus the FMEN1 gene functions as a tumor suppressor gene, analogous to the retinoblastoma gene. Analysis of sporadic parathyroid adenomas revealed that 25% showed allelic loss in a similar region. Thus the clonal inactivation of the FMEN1 gene may be a contributing factor in many sporadic parathyroid adenomas.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 43200-10 MDB
PERIOD COVERED October 1, 1988 through September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Disorders of Immune Regulation in Patients with Systemic Lupus Erythematosus		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P. I.: Others:	G. C. Tsokos J. E. Balow D. T. Boumpas H. Yamada	Guest Researcher MDB, NIDDK Senior Investigator MDB, NIDDK Visiting Associate MDB, NIDDK Visiting Fellow MDB, NIDDK
COOPERATING UNITS (if any) CC (A. Patel, Biologist)		
LAB/BRANCH Metabolic Diseases Branch		
SECTION Kidney Disease Section		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 2.00	PROFESSIONAL: 1.75	OTHER: 0.25
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>             Patients with systemic lupus erythematosus have been found to have various disturbances of the cell-mediated immune response. Cellular aberrations include enhanced spontaneous B lymphocyte activity with abnormal triggering in vitro, deficient immunoregulatory T lymphocyte circuits, deficient cytotoxic responses, including natural killer cell activity, alloantigen and viral cytotoxicity, and abnormal production of and response to different lymphokines as well as increased expression of proto-oncogenes in highly activated peripheral blood lymphocytes.           </p> <p>             CD4+ and CD4/CD8+ T lymphocyte receptor cells and cell lines from patients with active lupus nephritis provide help to autologous B lymphocytes to produce nephritogenic antibodies. The goal of these studies is to further elucidate the mechanisms of these alterations of the immune system which are apparently involved in the pathogenesis of this disease.           </p> <p>             The modulation of the above disturbances by immunosuppressive agents, i.e. corticosteroids and cyclophosphamide, is actively studied, aiming at the restoration of normal immune status in these patients.           </p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 43201-05 MDB
PERIOD COVERED October 1, 1988 through September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Production and Characterization of Nephritic Factors		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P. I.:	G. C. Tsokos	Guest Researcher      MDB, NIDDK
Others:	G. Thyphronitis	Visiting Fellow      MDB, NIDDK
	J. E. Balow	Senior Investigator      MDB, NIDDK
	E. D. Anastassiou	IRTA Fellow      MDB, NIDDK
COOPERATING UNITS (if any) SUNY Medical Center, Syracuse, NY (Dr. R. Spitzer); CC (A. Patel, Biologist).		
LAB/BRANCH Metabolic Diseases Branch		
SECTION Kidney Disease Section		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.00	0.75	0.25
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>             The nephritic factor (NeF) of the alternative pathway of complement has been found in the sera of patients with membranoproliferative glomerulonephritis (MPGN) and partial lipodystrophy and has been described as a factor which is able to induce cleavage of the third component of complement (C3) in normal human serum through the alternative pathway. It has been demonstrated that NeF binds to and stabilizes C3bBb (alternative C3 convertase). NeF appears to be antigenically and structurally similar to IgG and therefore it might be an autoantibody directed against C3bBb complex. The relation between the development of renal lesions and the NeF mediated persistent hypocomplementemia remains unexplained. B lymphocytes from patients with MPGN were used to establish cell lines secreting NeF of either IgG or IgM classes. Sera of patients with MPGN were found contain anti-idiotypic antibodies to NeF. We isolated 3 different anti-idiotypic antibodies and found that monoclonal and several polyclonal NeF share at least one idiotope. To verify this observation we are in the process of repeating these experiments using hetero-anti-idiotypic antibodies. Nucleotide sequencing of different NeF will answer the question whether NeF are direct products of germline genes or have undergone mutations as a result of antigenic stimulation.           </p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 43202-06 MDB
PERIOD COVERED October 1, 1988 through September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Regulation of Human Immune Response by Complement		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P. I.: Others:	G. C. Tsokos G. Thyphronitis J. E. Balow E. D. Anastassiou	Guest Researcher Visiting Fellow Senior Investigator IRTA Fellow  MDB, NIDDK MDB, NIDDK MDB, NIDDK MDB, NIDDK
COOPERATING UNITS (if any)		
LAB/BRANCH Metabolic Diseases Branch		
SECTION Kidney Disease Section		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 1.25	PROFESSIONAL: 1.25	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>Complement factors and breakdown products acting through cell surface membrane receptors block the differentiation of human B lymphocytes into immunoglobulin secreting cells. Complement receptors are associated with B cell surface immunoglobulin under certain circumstances. Furthermore, complement receptor expression is cell-cycle dependent.</p> <p>Monovalent ligands inhibit while polyvalent enhance the anti-IgM induced human B cell increase in intracytoplasmic Ca<sup>2+</sup> concentration and proliferation.</p> <p>Understanding of the mechanism of regulation of immune responses by complement and the role of complement receptors on human B cells is crucial for the understanding of the immunopathogenesis of autoimmune diseases since they are frequently associated with complement activation, depression of complement factor levels and changes in complement receptors.</p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 43204 C9 MDB
PERIOD COVERED October 1, 1988 through September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Immunosuppressive Drug Therapy in Lupus Glomerulonephritis		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	J. E. Balow	Senior Investigator
Others:	H. A. Austin	Medical Officer
		MDB, NIDDK
		MDB, NIDDK
COOPERATING UNITS (if any)  NIAMS (J. H. Klippel, P. H. Plotz, A. D. Steinberg, R. Wilder).		
LAB/BRANCH Metabolic Diseases Branch		
SECTION Kidney Disease Section		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 2.25	PROFESSIONAL: 1.50	OTHER: 0.75
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input checked="" type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>             The optimal treatment of the proliferative forms of kidney disease associated with systemic lupus erythematosus is controversial. The efficacy of intensive, intermittent immunosuppressive drug therapy is being evaluated in patients with active lupus glomerulonephritis. A comparison is being made between intermittent pulse doses of corticosteroid and cyclophosphamide, as well as between a short and long course of pulse cyclophosphamide. Patients with renal biopsy documented active glomerulonephritis are treated with prednisone and randomized to receive concomitantly (a) intravenous pulse methylprednisolone monthly for 6 months, or (b) intravenous pulse cyclophosphamide monthly for 6 months, or (c) pulse cyclophosphamide monthly for 6 months followed by a maintenance regimen of pulse cyclophosphamide every 3 months for an additional two years. During the final 24 months of the study, all patients continue to receive low dose, alternate day prednisone. Active disease, as manifested by renal functional deterioration, increased proteinuria or worsened urinary sediment, is treated by increased prednisone. Comparison will be made of the number of favorable outcomes of renal function, glomerular pathology and drug related toxicities occurring in each treatment.           </p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 43205-12 MDB
PERIOD COVERED October 1, 1988 through September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Renal Biopsy Pathology in Systemic Lupus Erythematosus		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: J. E. Balow	Senior Investigator	MDB, NIDDK
Others: H. A. Austin	Medical Officer	MDB, NIDDK
COOPERATING UNITS (if any)  Clinical Center (D. E. Webb); Armed Forces Institute of Pathology, Washington, DC (T. Antonovych, S. Sabnis).		
LAB/BRANCH Metabolic Diseases Branch		
SECTION Kidney Disease Section		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS:  0.25	PROFESSIONAL:  0.25	OTHER:
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input checked="" type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>             Diverse pathogenetic factors are operant in systemic lupus erythematosus and lead to different forms of lupus nephritis. Detailed analysis of renal biopsy pathology is being conducted on specimens from patients with systemic lupus erythematosus. Biopsies are classified by standard major category of lupus nephritis, as well as scored on a semiquantitative scale for specific histologic changes which indicates the extent and severity of active inflammatory lesions and of chronic atrophic, fibrosing and sclerosing features. The patterns of immune complex deposition and lymphoid cell interaction with different segments of the nephron are being investigated by immunohistologic techniques and electron microscopy. Computer-based morphometric techniques are being developed to analyze more precisely the changes in renal pathology during the course of lupus nephritis. These approaches have facilitated the analysis of the effects of various types of immunosuppressive agents used to halt the progression of lupus nephritis and they will enhance our understanding of the pathogenesis of the kidney disease of systemic lupus erythematosus.           </p>		

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43210-05 MDB

## PERIOD COVERED

October 1, 1988 through September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Glomerular Disease in Transgenic Mice

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	K. MacKay	Medical Staff Fellow	MDB, NIDDK
Others:	G. Striker	Director	DKUHD, NIDDK
	L. Striker	Expert	MDB, NIDDK

## COOPERATING UNITS (if any)

School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania (R. Brinster).

## LAB/BRANCH

Metabolic Diseases Branch

## UNIT

Renal Cell Biology

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

.6

## PROFESSIONAL:

.6

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- |   |  |   |
|---|--|---|
| <input type="checkbox"/> (a) Human subjects | <input type="checkbox"/> (b) Human tissues | <input checked="" type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors        |  |   |
| <input type="checkbox"/> (a2) Interviews    |  |   |

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Mice transgenic for SV40 develop progressive glomerulosclerosis. We have postulated that the disease is due to an abnormal proliferative stimulus (T antigen) leading to mesangial proliferation. We are now investigating whether a stimulus known to induce renal hypertrophy results in an acceleration of the glomerular lesions. To that effect we have performed unilateral nephrectomies in six week old SV40 mice and are examining the expression of T antigen and growth factors (IGF-I, TGF-B).

We have performed nephrectomies on SV 40 mice to assess the role of a stimulus for compensatory hypertrophy and found an acceleration of the glomerulosclerosis in female mice.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43211-05 MDB

## PERIOD COVERED

October 1, 1988 through September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Histopathology of Renal Lesions in Pima Indians

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	L. Striker	Expert	MDB, NIDDK
Others:	G. Striker	Director	DKUHD, NIDDK

## COOPERATING UNITS (if any)

Epidemiology and Clinical Research Branch, NIDDK, Phoenix, Arizona  
(P. Bennett).

## LAB/BRANCH

Metabolic Diseases Branch

## UNIT

Renal Cell Biology

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

.3

## PROFESSIONAL:

.3

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- |   |   |                                      |
|---|---|--------------------------------------|
| <input type="checkbox"/> (a) Human subjects | <input checked="" type="checkbox"/> (b) Human tissues | <input type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors        |   |                                      |
| <input type="checkbox"/> (a2) Interviews    |   |                                      |

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Autopsies from diabetic and non-diabetic Pima Indians will be examined from a series drawn as a representative sample of the autopsy population. Routine light microscopic studies, and potentially electron microscopic studies, will be performed to assess the histopathologic lesions present in these autopsy specimens. Particular attention will be paid to epithelial basement membranes and vascular extracellular matrix areas.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 43214-05 MDB

PERIOD COVERED

October 1, 1988 through September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cell and Molecular Biology of Glomerular Cells Derived From Transgenic Mice

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	K. MacKay	Medical Staff Fellow	MDB, NIDDK
Others:	L. Striker	Expert	MDB, NIDDK
	G. Striker	Director	DKUHD, NIDDK
	S. Elliot	Biologist	MDB, NIDDK

COOPERATING UNITS (if any)

School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania (R. Brinster).

LAB/BRANCH

Metabolic Diseases Branch

UNIT

Renal Cell Biology

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project is inactive.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43217-05 MDB

## PERIOD COVERED

October 1, 1988 through September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Renal Lesions in Leukemias, Lymphomas and Carcinomas

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	L. Striker	Expert	MDB, NIDDK
Others:	G. Striker	Director	DKUHD, NIDDK
	L. Agodoa	Medical Officer	MDB, NIDDK

## COOPERATING UNITS (if any)

National Cancer Institute, Bethesda, Maryland (M. Merino, W. Travis).

## LAB/BRANCH

Metabolic Diseases Branch

## UNIT

Renal Cell Biology

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

.5

## PROFESSIONAL:

.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We evaluated glomerular lesions in kidneys from patients who underwent nephrectomy for renal cancer. In areas non-invaded by the tumor there was, in one-half of the cases examined, marked mesangial proliferation and occasional synechiae. This suggested that the glomerular lesions we found could be mediated by growth factors released from the tumor.

In a separate group of patients we examined the effect of interleukin II on the kidneys of 19 patients who died in the Clinical Center. The occurrence of lesions was correlated with the creatinine and BUN level.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43220-04 MDB

## PERIOD COVERED

October 1, 1988 through March 31, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Expression of Angiotensin Converting Enzyme in Renal Glomeruli

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: L. Striker

Expert

MDB, NIDDK

## COOPERATING UNITS (if any)

National Institute of Mental Health, Bethesda, Maryland (B. Martin);  
Emory University, Atlanta, Georgia (K. Bernstein).

## LAB/BRANCH

Metabolic Diseases Branch

## UNIT

renal Cell Biology

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

.1

## PROFESSIONAL:

.1

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

It has been suggested on an experimental basis that elevated intraglomerular pressure leads to glomerulosclerosis. The regulation of angiotensin converting enzyme (ACE) production plays a central role in maintaining normal intraglomerular pressure. This project was designed to isolate the gene encoding ACE from mouse kidney to further study the regulation and expression of the enzyme.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43221-04 MDB

## PERIOD COVERED

October 1, 1988 through September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biology of Insulin Receptors in Glomerular Cells

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	S. Elliot	Biologist	MDB, NIDDK
Others:	F. Conti	Visiting Fellow	MDB, NIDDK
	G. Striker	Director	DKUHD, NIDDK
	L. Striker	Expert	MDB, NIDDK
	T. Doi	Visiting Associate	MDB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Metabolic Diseases Branch

## UNIT

Renal Cell Biology

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

.8

## PROFESSIONAL:

.8

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

We propose to study insulin specific binding on glomeruli from mice and humans. Binding of insulin to mesangial cells from normal mice is being investigated. The nature of the receptor will be studied and elucidated. Binding of insulin to an endothelial clone derived from normal mouse glomeruli has been shown. The eventual role of insulin as a progression factor is being investigated.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 43222-04 MDB
PERIOD COVERED October 1, 1988 through September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Pathogenesis of Murine Lupus Nephritis		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <div style="display: flex; justify-content: space-between; margin-top: 10px;"> <div style="width: 30%;"> <b>P. I.:</b> H. A. Austin  <b>Others:</b> J. E. Balow            C. Cadena         </div> <div style="width: 30%;"> <b>Medical Officer</b>  <b>Senior Investigator</b>  <b>Biologist</b> </div> <div style="width: 30%;"> <b>MDB, NIDDK</b>  <b>MDB, NIDDK</b>  <b>MDB, NIDDK</b> </div> </div>		
COOPERATING UNITS (if any) Armed Forces Institute of Pathology; Washington, DC (Drs. T. Antonovych and S. Sabnis).		
LAB/BRANCH Metabolic Diseases Branch		
SECTION Kidney Disease Section		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 1.50	PROFESSIONAL: 0.50	OTHER: 1.00
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div> <input type="checkbox"/> (a) Human subjects  <input type="checkbox"/> (a1) Minors  <input type="checkbox"/> (a2) Interviews         </div> <div> <input type="checkbox"/> (b) Human tissues         </div> <div> <input checked="" type="checkbox"/> (c) Neither         </div> </div>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <div style="margin-top: 20px;"> <p>Investigations of the pathogenesis and treatment of lupus nephritis are facilitated by the availability of inbred strains of mice that develop disease similar to human systemic lupus erythematosus. The natural evolution of the diverse histologic features of murine lupus nephritis is being studied to delineate the types of glomerular and tubulointerstitial lesions. Monoclonal antibodies and immunoperoxidase staining of frozen sections are employed to study the types and distribution of lymphoid cells in glomerular, vascular and tubulointerstitial lesions. Changes in mesangial clearance of a macromolecular tracer, ferritin, will be related to these features of progressive glomerular disease. The impact of biologic response modifiers on serologic and renal histologic features is being investigated. The goal is to develop a model of a flare of lupus nephritis which would facilitate further investigations of immunopathogenic mechanisms. Innovative treatment strategies will be studied to refine our approach to this disease. Clinical, histologic and immunologic outcome parameters will be evaluated including detailed studies of renal morphology, and the characteristics of peripheral blood lymphocytes and splenocytes employing flow cytometry and in vitro assays of alterations in humoral and cell mediated immune regulation.</p> </div>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 43224-03 MDB
PERIOD COVERED October 1, 1988 through September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Membranous Lupus Nephropathy		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: J. E. Balow Others: H. A. Austin	Senior Investigator Medical Officer	MDB, NIDDK MDB, NIDDK
COOPERATING UNITS (if any) CC (D. Webb, K. Joyce, E. Vaughan); NIAMS (J. Klippel); Stanford University; Stanford, CA (B. Myers).		
LAB/BRANCH Metabolic Diseases Branch		
SECTION Kidney Disease Section		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 2.00	PROFESSIONAL: 1.50	OTHER: 0.50
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input checked="" type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>It is currently unknown whether therapeutic intervention will alter the course of membranous lupus nephropathy. In the present study, the efficacy and toxicity of three immunosuppressive drug regimens administered over a 12 month period will be evaluated in patients with membranous lupus nephropathy. Detailed tests of renal function (including radiolabelled compounds for glomerular filtration and renal plasma flow rates), glomerular permselectivity (using fractional clearance of graded dextrans) and kidney biopsy morphology will be performed at the beginning and end of treatment. Patients with systemic lupus erythematosus, nephrotic range proteinuria and biopsy documented membranous nephropathy will be randomized to receive: a) alternate day prednisone alone (control group), b) alternate day prednisone plus intravenous pulse cyclophosphamide up to 1.0 gram per square meter body surface area every other month for 6 total doses, or c) alternate day prednisone plus oral cyclosporin A up to 200 mg per square meter body surface area daily. Lupus disease activity, renal function tests and drug toxicities will be monitored closely. Analysis will include comparison of the numbers of favorable outcomes of glomerular filtration rate, renal plasma flow, permselectivity, glomerular pathology and drug-related toxicities appearing in each treatment group.</p>		

## NOTICE OF INTRAMURAL RESEARCH PROJECT

201 DK 43225-02 MDB

## PERIOD COVERED

October 1, 1988 through September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Glomerular Changes Due to GH and IGF-I

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: L. Striker Expert MDB, NIDDK

Others: T. Doi Visiting Associate MDB, NIDDK

L. Agodoa Medical Officer MDB, NIDDK

F. Conti Visiting Fellow MDB, NIDDK

## COOPERATING UNITS (if any)

University of Washington, Seattle, Washington (R. Palmiter); School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania (R. Brinster).

## LAB/BRANCH

Metabolic Diseases Branch

## UNIT

Renal Cell Biology

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

.9

## PROFESSIONAL:

.9

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Increased glomerular size occurs in the presence of normal maturation following unilateral nephrectomy in humans and animals and in disease states such as diabetes mellitus. The glomeruli are morphologically and functionally normal following nephrectomy in rats unless the remaining renal mass is severely reduced, in which case progressive glomerulosclerosis ensues. The hormonal regulation of compensatory hypertrophy is not fully understood, however total kidney IGF-I mRNA levels are increased following unilateral nephrectomy. This suggests a role for this hormone in hypertrophy of the adult kidney as well as in normal development. There are abnormalities in the circulating levels of GH in some diseases associated with increases in glomerular extracellular matrix and cell number such as diabetes mellitus. The availability of transgenic mouse strains expressing elevated levels of IGF-I, GH, and GHRF provides an opportunity to study the renal effects of chronic hormone exposure. We have observed that mice containing an MT-I IGF-I fusion gene develop large glomeruli which are normal in appearance, whereas those transgenic for either growth hormone or growth hormone releasing factor have large glomeruli which are hypercellular, whereas progressive glomerulosclerosis develops later.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43226-02 MDB

## PERIOD COVERED

October 1, 1988 through September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effect of TGF-B on Glomerular Cells

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	K. MacKay	Medical Staff Fellow	MDB, NIDDK
Others:	L. Striker	Expert	MDB, NIDDK
	G. Striker	Director	DKUHD, NIDDK
	L. Agodoa	Medical Officer	MDB, NIDDK
	T. Doi	Visiting Associate	MDB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Metabolic Diseases Branch

## UNIT

Renal Cell Biology

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- |   |  |   |
|---|--|---|
| <input type="checkbox"/> (a) Human subjects | <input type="checkbox"/> (b) Human tissues | <input checked="" type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors        |  |   |
| <input type="checkbox"/> (a2) Interviews    |  |   |

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Proliferation of resident glomerular cells and the accumulation of mesangial matrix are histologic abnormalities which are observed in the course of many progressive glomerular diseases. We explored the potential regulatory effects of transforming growth factor-B (TGF-B) on these processes. We found that cultured mouse glomerular endothelial, mesangial, and epithelial cells as well as isolated rat glomeruli possess high affinity receptors for TGF-B. We also found that while TGF-B consistently inhibited the proliferation of glomerular endothelial and epithelial cells it acted as a bifunctional regulator of mesangial cell proliferation. The presence of TGF-B receptors on each glomerular cell type and on isolated glomeruli, the multiple potential sources of TGF-B within the glomerulus and the demonstrated responsiveness of cultured glomerular cells to TGF-B convine to suggest that potentially important interactions may occur between resident glomerular cells and TGF-B in vivo.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43227-02 MDB

## PERIOD COVERED

October 1, 1988 through September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Role of IGF-I in the Biology of Mouse Glomerular Cells

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	F. Conti	Visiting Fellow	MDB, NIDDK
Others:	T. Doi	Visiting Associate	MDB, NIDDK
	S. Elliot	Biologist	MDB, NIDDK
	L. Striker	Expert	MDB, NIDDK
	G. Striker	Director	DKUHD, NIDDK

## COOPERATING UNITS (if any)

Diabetes Branch, NIDDK (M. Lesniak, J. Roth).

## LAB/BRANCH

Metabolic Diseases Branch

## UNIT

Renal Cell Biology

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The kidney has been shown to be a site of production of IGF-I in the rat, mouse and human in many experimental conditions. IGF-I mRNA and peptide were found to increase in renal compensatory hypertrophy following partial nephrectomy suggesting a role of this peptide in renal growth. However the sites of action and the cell types responsible for IGF-I synthesis have not been identified. To further investigate the role of IGF-I in the renal glomerulus we used mouse glomerular cells in culture. Separate cultures of glomerular endothelial, epithelial and mesangial cells were obtained from isolated glomeruli using patch cloning and dilute plating techniques. These cells were studied for the presence of IGF-I receptors, the mitogenic response to IGF-I and the synthesis of this growth factor. Glomerular mesangial cells were found to have IGF-I receptors and to be sensitive to the mitogenic action of IGF-I. These cells were also found to synthesize and release in the culture medium an IGF-I like molecule. Glomerular endothelial and epithelial cells were found to have IGF-I receptors, but no IGF-I like molecule was detected in their culture medium. These data suggest a role for IGF-I in the maintenance and regulation of kidney structure and function. They demonstrate that glomerular mesangial cells may be a source of IGF-I in the kidney. IGF-I might be one of the growth factors locally released in the glomerulus acting in an autocrine and paracrine fashion.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43228-02 MDB

## PERIOD COVERED

October 1, 1988 through September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biology of Human Glomerular Mesangial Cells

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: L. Striker Expert MDB, NIDDK

Others: T. Doi Visiting Associate MDB, NIDDK  
S. Elliot Biologist MDB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Metabolic Diseases Branch

## UNIT

Renal Cell Biology

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

.5

## PROFESSIONAL:

.5

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Mesangial cell hyperplasia is a feature common to several human glomerular diseases. The cause of this increased cell number is unknown. We assessed human mesangial cells in vitro and found that they possessed an insulin-like growth factor-I (IGF-I) receptor consisting of  $\alpha$  and  $\beta$  units (Mr 130k and 90k respectively). Fifty percent inhibition of IGF-I specific binding to the receptor required  $1 \times 10^{-9}$ M IGF-I,  $\geq 1 \times 10^{-6}$ M insulin and  $1 \times 10^{-7}$ M multiplication stimulating activity (MSA). Analysis of binding by the method of Scatchard revealed one type of IGF-I receptor with a  $K_d = 1.35 \times 10^{-9}$ M, and a number per cell of  $1.04 \times 10^5$ . Binding studies on whole glomeruli was of similar specificity and there were  $7.17 \times 10^7$  receptors per glomerulus ( $K_d = 1.12 \times 10^{-9}$ M). Examination of the effect of IGF-I on the cell cycle revealed that cells treated with platelet derived growth factor (PDGF) had a rapid  $^3\text{H}$ -thymidine response which was abolished by anti-PDGF antibody. Similarly, the labeling index of cells pretreated with PDGF, washed and then exposed to IGF-I was increased, whereas if the order of ligand exposure was reversed, there was no such additive effect. Finally, PDGF increased RNA and protein synthesis, which was not enhanced by IGF-I. In summary, human mesangial cells, and whole glomeruli, possess IGF-I specific receptors and IGF-I was found to act as a progression factor in the cell cycle.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43229-01 MDB

## PERIOD COVERED

May 24, 1988 through August 31, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Binding and Uptake of Mouse IgA by Mouse Glomerular Mesangial Cells

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: L. Agodoa Medical Officer MDB, NIDDK

Others: S. Elliot Biologist MDB, NIDDK  
L. Striker Expert MDB, NIDDK

## COOPERATING UNITS (if any)

Brown University, Providence, Rhode Island (R. Rifai).

## LAB/BRANCH

Metabolic Diseases Branch

## UNIT

Renal Cell Biology

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

.7

## PROFESSIONAL:

.7

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

IgA nephropathy is probably the most common form of human glomerulonephritis. Experimental IgA nephropathy, similar to the human disease, can be induced in mice using cross-linked DNP and mouse IgA anti-DNP. The mechanism of binding of IgA by mesangial cells was studied using mouse glomerular mesangial cells in vitro.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 43230-01 MDB

## PERIOD COVERED

October 1, 1988 through September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Proteoglycan Production by Mouse Glomerular Epithelial Cells

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: G. Striker Director DKUHD, NIDDK

Others: K. MacKay Medical Staff Fellow MDB, NIDDK  
L. Striker Expert MDB, NIDDK

## COOPERATING UNITS (if any)

Department of Cell Biology, Yale School of Medicine, New Haven, Connecticut  
(J. Stow, M. Farquhar).

## LAB/BRANCH

Metabolic Diseases Branch

## UNIT

Renal Cell Biology

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

.6

## PROFESSIONAL:

.6

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Mouse glomerular epithelial cells previously characterized have been investigated for biosynthesis of proteoglycans. Confluent monolayers of epithelial cells were radiolabeled. It was found that these cells produced Heparan Sulfate basement membrane in high amounts. Analysis of immunoprecipitates showed both a mature proteoglycan and a precursor core protein band. These proteoglycans are an important determinant of the permselectivity properties of the glomerular basement membrane.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 43231-01 MDE
PERIOD COVERED October 1, 1988 through September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Idiopathic Membranous Nephropathy		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)  P. I. : H. A. Austin Medical Officer MDB, NIDDK Others: J. E. Balow Senior Investigator MDB, NIDDK		
COOPERATING UNITS (if any) Stanford University, Stanford, CA (Dr. B. Myers); CC (K. Joyce, E. Vaughan, Nursing); Armed Forces Institute of Pathology, Washington, DC (Drs. T. Antonovych and S. Sabnis).		
LAB/BRANCH Metabolic Diseases Branch		
SECTION Kidney Disease Section		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 1.00	PROFESSIONAL: 0.50	OTHER: 0.50
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input checked="" type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>             Patients with idiopathic membranous nephropathy are being studied to evaluate the efficacy and toxicities of the addition of intermittent cyclophosphamide or daily oral cyclosporin A to alternate day oral corticosteroid therapy. Efficacy will be judged by determinations of effective renal plasma flow, glomerular filtration rate and glomerular capillary wall permselectivity performed with dextran and urine protein (albumin and immunoglobulin) clearance techniques. Kidney biopsy morphology (including morphometric analysis) will be examined at the beginning and at the end of treatment as part of detailed studies of structure-function relationships and the efficacy of various therapeutic modalities. Patients with membranous nephropathy and 2 or more grams per day of proteinuria will be treated with alternate day prednisone and will be randomized to receive: a) no additional therapy (control group), b) intravenous pulse cyclophosphamide up to 1.0 gram per square meter body surface area every other month for 6 total doses, or c) oral cyclosporin A up to 200 mg per square meter body surface area daily for a total of 11 months. Analysis will include comparison of the number of favorable outcomes of glomerular function and pathology as well as drug-related toxicities observed in each treatment group at the end of the 12 months of study.           </p>		

Annual Report of the Clinical Endocrinology Branch  
National Institute of Diabetes and Digestive and Kidney Diseases

The Third Edelhoch Memorial Lecture in the series: Endocrine Grand Rounds, was delivered by Dr. Ira Pastan of the National Cancer Institute, a former member of the Branch and a colleague of Dr. Edelhoch. His topic was on the targeting of toxins to tumor cells through the fusion of growth factors and toxic genes.

Dr. Sheryl Sato, a new investigator in the Branch, arrived in May 1989. She is currently completing experiments under way in NICHD where she has been a fellow under Dr. Igor David. Her major field of interest will be the differentiation of endocrine organs.

A number of countries were represented by the visiting fellows and scientists in the Branch. These included France, Greece, India, Italy, and Japan.

I. Thyroid Biochemistry and Pathophysiology

A. Thyroid Hormone-Protein Interactions

Previous work demonstrated that a minor component of the circulating thyroid hormones can be attributed to thyroxine (T<sub>4</sub>) and 3, 5, 3'-triiodothyronine (T<sub>3</sub>) bound to the three classes of lipoproteins, VLDL, HDL, and LDL. It was further shown that this is the result of specific interaction with apolipoproteins: apo A-I and apo C-II or III in HDL, apo B-100 in LDL. To test the hypothesis that these complexes might have a role in transporting thyroid hormones into cells, it was elected to first examine the

effect of LDL in fibroblasts since cells that are totally deficient in LDL receptors are available from patients with homozygous familial hypercholesterolemia. Preliminary experiments showed that normal and LDL receptor-deficient fibroblasts accumulated T<sub>4</sub> and T<sub>3</sub> from serum-free medium by a specific, energy-dependent process with no difference between the cell types. When normal fibroblasts were grown in lipoprotein deficient serum in order to maximize their surface LDL receptors, and then tested for the effect of LDL on saturable T<sub>4</sub> uptake, it was found that both the initial rate of T<sub>4</sub> uptake and the equilibrium value at 60 minutes were increased as much as 2-fold. The amount of the increase was proportional to the concentration of LDL until LDL receptors were saturated. At higher levels, LDL inhibited uptake by competing with the cell surface T<sub>4</sub> receptors for hormone. Isolated apoB-100 and apoE also facilitated T<sub>4</sub> uptake, as expected from the fact that LDL receptors recognize both apolipoproteins. LDL receptor-deficient fibroblasts, and normal fibroblasts whose LDL receptors were down regulated by culture in 10% fetal bovine serum, showed no effect of LDL on T<sub>4</sub> uptake. LDL had no effect on T<sub>3</sub> uptake, attributable to the fact that the affinity of T<sub>3</sub> for LDL is one-tenth that of T<sub>4</sub>.

These experiments show that T<sub>4</sub> uptake by LDL-competent fibroblasts is enhanced by binding to the apoprotein moiety of LDL presumably during receptor mediated endocytosis of the lipoprotein. Since virtually all cell types express LDL receptors, the increased T<sub>4</sub> entry promoted by LDL should

be of general significance, and may have a role in the intracellular targeting of the hormone. (Robbins, Benvenaga, Cahnmann).

#### B. Thyroid Hormone Metabolism

As described above, both T<sub>4</sub> and T<sub>3</sub> are specifically transported into human fibroblasts in the absence of serum. Our earlier work similarly showed that T<sub>4</sub> and T<sub>3</sub> are actively accumulated by cells of the central nervous system (glioma and neuroblastoma), but we have been unable to demonstrate such uptake of T<sub>4</sub> in skeletal muscle. Further studies were performed with rat skeletal myoblasts, a cell line that possesses a saturable, energy-dependent mechanism for T<sub>3</sub> entry. It was shown that the uptake of T<sub>4</sub> was not saturable at any temperature, and that uptake of L-T<sub>4</sub> did not differ from that of D-T<sub>4</sub>. Furthermore, preloading the cells with a high T<sub>4</sub> concentration (1  $\mu$ M) did not alter the initial rate of T<sub>4</sub> uptake, in keeping into a simple diffusive process, and inhibitors of energy production, Na<sup>+</sup>-K<sup>+</sup> ATPase and endocytosis had no effect. Thus, it is apparent that separate processes for T<sub>4</sub> and T<sub>3</sub> entry may exist in different types of cells. It is of interest that nervous system cells are especially dependent on T<sub>4</sub> entry since most of their nuclear receptor-bound T<sub>3</sub> is derived from intracellular monodeiodination of T<sub>4</sub> (Robbins, DiCerbo, Pontecorvi, Goncalves).

#### C. Thyroid Hormone Action

In studies reported last year, it was shown that variant forms of the alpha c-erb-A mRNA are expressed at high level in rat brain, accounting for the discrepancy between the high content of c-erbA-related mRNA and the known low content of T<sub>3</sub> nuclear receptor in brain. It was concluded that the  $\alpha$  receptor protein is encoded by 5.4 and 6.8-kb species and that variant proteins are encoded by 2.6-kb mRNA. Analysis of the 3' structures of the mRNA disclosed a large difference in length between the reported  $\alpha$  receptor DNA and the mRNAs detected by a receptor cDNA-specific probe. The mRNA size difference could be ascribed to the presence of a large 3'-untranslated region and the utilization of different polyadenylation sites. Analysis of genomic clones revealed a canonical polyadenylation signal (AATAAA) (PA1) at the 3' end of region x and a noncanonical signal (PA2) at the 3'-end of region d. GT-rich regions were found just downstream from these signals, as expected for generating the 3' end of mRNA. The first polyadenylation signal gives rise to the 5.4-kb mRNA and the second gives the 6.8-kb species.

RNAse protection assays showed that the ratios of normal receptor  $\alpha$  mRNA to variant mRNA varied in different tissues, being highest in brain and kidney and lowest in liver, heart, and spleen. The expression pattern during development of the different forms of mRNA was studied by Northern analysis. In fetal brain (day 19) the 5.4 and 6.8-kb species were barely detectable, and increased slightly by 5 to 10 days after birth. In adult brain, the 5.4-kb mRNA became predominant. On the other hand, the expression level of the 2.6-kb mRNA encoding the variant proteins remained unchanged during development.

Expression of the alternative mRNAs was also examined in various thyroid states. In brain, no discernable changes were found between hyper-, eu-, and hypothyroidism. In liver, however, the level of all mRNA

forms were highest in hypothyroidism and were decreased by 50% in hyperthyroidism. Knowledge of these variation in expression should aid in the understanding of how the coding capacity of single genes are regulated. (Nikodem, Mitsuhashi).

Last year, it was reported that in a thyroid hormone-responsive tissue, rat liver, T3 caused an increase in binding of a trans-acting promoter protein to two cis-regulatory elements of the malic enzyme (ME) gene located at -144/-123 and -73/-50. Their role was defined more precisely by transcription competition analysis in which an excess of oligonucleotide is added to the transcription reaction to effectively remove any trans-acting factors that bind to that DNA sequence. The intact malic enzyme promoter in pME-cat was transcribed by HeLa cell extracts in the absence or presence of a series of synthetic oligonucleotides. A 200-fold molar excess of an oligomer containing a 10 base pair direct repeat (regulatory element -73/-50) and an oligomer containing sequences of AP-1 binding site (element -144/-123) produced > 85% inhibition of transcription.

These oligonucleotides were also used to quantitate binding of trans-acting factors in liver nuclear extracts. Binding showed a marked dependence on the thyroid status of rats used to prepare the liver extracts. Each oligomer interacted with at least two protein bands that were reduced in hypothyroid liver and enhanced in hyperthyroid liver. In contrast, nuclear extracts from testis, an organ in which the ME gene is not regulated by thyroid hormone, showed no effect of thyroid status on binding to the oligonucleotides.

The time course of changes in the trans-acting factors in liver nuclei in response to T3 treatment was compared with the corresponding rates of transcription of the malic enzyme gene. Binding to the oligomer containing the AP-1 site was maximally induced at 1 day of T3 treatment and that containing the direct repeat motif was markedly elevated at 1 day and maximal after 6 days. The results demonstrated that the two trans-acting factors were induced slightly in advance of changes in the transcription rate of the gene, a finding that supports their involvement as mediators in the induction of transcription of the ME gene by T3. (Nikodem, Petty, Mitsuhashi, Morioka).

To study the interaction of T3 receptors with the 5' flanking region of the ME gene, coding regions of the T3 receptor (c-erbA), rat alpha (rTR $\alpha$ ) and human beta (hTR $\beta$ ) were transcribed and translated in vitro. Gel shift assay showed specific binding of both to ME -354/-178 but not to ME -177/+33. Competition binding analysis with synthetic oligonucleotides demonstrated high affinity binding of both T3 receptors in the presence or absence of T3. Transfection experiments with NIH 3T3 and COS7 cells, however, showed that T3 is required to increase the basal level of expression ( $\approx 3.5$  fold) only of the ME-CAT construct -354/+33 when cotransfected with plasmids containing either rTR $\alpha$  or hTR $\beta$ . Thus, transcriptional activation of the ME gene by T3 is mediated primarily by the T3 receptor, and is modulated by the binding of other trans-acting factors responding to T3. (Nikodem, Desvergne, Petty, Mitsuhashi).

The adrenal hormone, dehydroepiandrosterone (DHEA) is known to stimulate hepatic malic enzyme activity in rats. The mechanism of this effect, and its relation to the control of ME activity by thyroid hormones,

was investigated. Using the intronic ME probe previously developed, it was shown that DHEA increased the rate of transcription of the ME gene in a dose-dependent manner, up to 8-9 fold. This increase was specific for liver and was not seen in brain, heart, kidney or testis. The increase in transcription rate accounted for the observed increase in ME mRNA and ME activity, ruling out an effect on RNA stability as observed with T3. Thyroid hormone was absolutely required for the expression of the DHEA. These stimuli had additive effects on the level of ME mRNA. In conjunction with previous work on the response of ME activity to thyroid hormone and carbohydrate feeding, it is evident that ME gene expression is regulated by different mechanisms depending on the inducing factor. (Nikodem, Song, Grieco, Rall)

Fetal and neonatal thyroid status affects the growth, development and maturation of the mammalian central nervous system. These processes are dependent on the presence of thyroid hormone during the so-called critical period, and their failure leads to irreversible brain damage. A prominent feature of this development is arborization of neural processes and their myelination. The molecular basis of myelinogenesis has recently been characterized in mice, facilitated by cloning of the genes for myelin proteolipid protein, glycoprotein Po, and myelin basic protein (MBP). The latter accounts for 30% of total myelin protein. The primary transcripts of a single MBP gene are alternatively spliced to generate mRNAs for 4 proteins of molecular mass  $\approx$ 14, 17, 18.5 and 21 kDa. The present study was initiated to define the regulatory role of thyroid hormone in MBP gene expression. Experiments completed thus far have confirmed previous findings in neonatal C57BL/6 mice and disclosed an unexpected effect of hypothyroidism in the adult.

Total RNA from whole brain was analyzed by Northern analysis and RNase protection assay. Expression of all 4 MBP mRNAs was detected at 4 days after birth, with higher expression of mRNAs coding for 21.5 and 17 kDa isoforms. Maximal expression was at 18 days after birth, with an increase in the proportion of 18.5 and 14 kDa species. In the adult, the 14 kDa form was predominant. Surprisingly, brains from hypothyroid adult mice had an 80% lower concentration of MBP mRNAs than euthyroid mice. Controls with mRNAs known not to be affected by thyroid status ( $\beta$ -actin, malic enzyme, thyroid hormone receptor) were not altered. Studies to define the mechanism of the hypothyroid effect are in progress. (Nikodem, Farsetti, Mitsuhashi, Robbins).

#### D. Studies in Thyroid Disease

Lithium cation and iodide anion have in common an effect on thyroid gland function not shared by any other known agent; i.e., the ability to inhibit secretion of thyroid hormone by a direct effect on the gland. Since the effect of both drugs is enhanced in a damaged thyroid, and since iodide has been successfully used to control hyperthyroidism in patients treated with low-dose radioiodine (I131), a study was conducted to compare the effects of  $\text{Li}^+$  with those of  $\text{I}^-$ . In 3 patients,  $\text{Li}^+$  and  $\text{I}^-$  were given alternately or in combination following I131 therapy for Graves' disease. It was found that  $\text{Li}^+$  was effective in controlling the hyperthyroid state, but at the doses used (300 mg  $\text{Li}_2\text{CO}_3$  tid)  $\text{Li}^+$  was less likely to induce hypothyroidism than  $\text{I}^-$  (43 mg tid). Combined therapy was more effective

than either agent alone. It was concluded that  $\text{Li}^+$  can be used as an alternative to  $\text{I}^-$  or antithyroid drugs to control hyperthyroidism after  $\text{I}^{131}$  therapy.

The effects of T3 versus T4 treatment, and thyroid hormone withdrawal, on mood and cognitive function was studied in patients who previously had a thyroidectomy for thyroid cancer. This collaborative study with NIMH made use of our thyroid protocol patients routinely undergoing a change in thyroid hormone status for diagnostic radioiodine scanning. The subjects included 25 patients who received sequentially T4, T3 for 2 weeks, and no thyroid replacement for 2 1/2 weeks. The studies revealed a change in mood when patients were off medication (increased sadness and anxiety), but no significant difference in mood was noted while patients were receiving T4 compared with T3. Thus, a brief period of hypothyroidism produced definite mood changes but the postulated differential effects of T4 and T3 were not observed. Those patients who experienced increased affective symptoms when off medication were more likely to be characterized by a personal history of affective illness or of mood lability.

Thirteen additional patients undergoing thyroid hormone withdrawal as just described were studied in collaboration with NINCDS to evaluate published opinions that hypothyroidism is associated with both increased and decreased blood pressure. At 2 1/2 weeks following withdrawal of T3, a small but significant decrease in systolic blood pressure occurred, both supine and standing, while the corresponding plasma norepinephrine levels increased significantly. These findings indicate that the acute cardiovascular effect of brief thyroid hormone withdrawal is a decrease in blood pressure rather than the increase often observed in chronic hypothyroidism, and that plasma norepinephrine levels may increase much sooner after onset of hypothyroidism than previously reported.

Other studies that are in progress on patients with thyroid cancer include the following: 1) The effect of lithium carbonate on the secretion rate of radioiodine by thyroid cancer metastases. The purpose of this study is to improve the risk/benefit ratio of radioiodine therapy. 2) The effect of lithium carbonate on the secretion rate of radioiodine from thyroid remnants remaining after initial surgery for thyroid cancer. The purpose of this study is to improve the yield of complete ablation by low dose (30 mCi)  $\text{I}^{131}$  ablation therapy. 3) The management of patients on renal dialysis during radioiodine scanning and therapy. The purpose is to design safe and effective therapy in thyroid cancer patients who lack normal renal handling of radioiodine. 4) Initiation of a randomized treatment protocol in high risk differentiated thyroid cancer to compare the effect of combined  $\text{I}^{131}$  + low dose adriamycin with  $\text{I}^{131}$  alone. (Robbins, Ain).

## II. Mechanism of Secretion

As reported previously, three experimental approaches have been employed in order to evaluate the domain structure of tubulin, the subunit protein of microtubules, that are intimately involved in the motility of cells and their organelles. These approaches have now led to a further elucidation of tubulin structure.

Limited proteolysis of the  $\alpha$  and  $\beta$  subunits of tubulin by subtilisin takes place at C-terminal cleavage sites and the rate constant is proportional to the ratio of subtilisin to tubulin. With decreasing tubulin concentration, the zero time intercept decreases, apparently due to creation of a rapidly digested fraction that represents monomeric  $\beta$ -tubulin formed by dimer dissociation. The concentration dependence indicates a dissociation constant of  $1.5 \times 10^{-7} \text{M}$ . This value was verified by rapid, short column equilibrium sedimentation in the analytical ultracentrifuge using a method developed for the purpose of making possible the analysis of small volumes ( $\approx 100 \mu\text{l}$ ) containing only a few  $\mu\text{g}$  of protein. This method allowed collection of  $\approx 500$  data points per  $100 \mu\text{l}$  sample within  $\approx 2$  min. The apparatus can be easily assembled from a commercially available data acquisition board, a standard personal computer, and an interface circuit enabling the final data fitting by a mainframe computer. The value obtained for the tubulin  $\alpha$ - $\beta$  dissociation constant was  $2 \times 10^{-7} \text{M}$ .

In the third approach, fluorescence measurements were made using the polarity-sensitive dye, nile red and time resolved spectral, quenching and anisotropy analysis. Binding of nile red to tubulin gave a blue shift in fluorescence emission and revealed two components with fluorescent lifetimes of 4.5 ns and 0.6 ns. Only the former was quenched by acrylamide. Ground state (excitation) heterogeneity was also shown and the more shielded site showed energy transfer from tryptophane. These data are consistent with a model in which the more "nonpolar" binding site is located in a region of subunit-subunit contact which is lost on dimer dissociation with a  $K_d$  of  $\approx 7 \times 10^{-7} \text{M}$ . [Wolff, Sackett, Knudsen (NHLBI)].

A study has been initiated to investigate the role of the cytoskeleton in secretion of adrenal hormone. Cholesterol was found to bind to tubulin. Since cholesterol is an intermediate in steroidogenesis, this binding may be of physiological importance. (Wolff, Sackett, Shiver).

### III. Adenylate Cyclase of Bacterial Origin

The virulence factor of Bordetella Pertussis that has been characterized as an extrabacterial adenylate cyclase has been further defined. In particular, studies are directed at establishing the mechanism by which the cyclase gains entry into cells of the animal host. It is the invasive form that is associated with bacterial toxicity. Experiments with highly purified enzyme have shown that the invasive and catalytic properties probably reside on the same molecule. Limited proteolysis by trypsin, chymotrypsin and subtilisin abolishes intracellular penetration without affecting cyclase activity. Although higher protease concentrations inhibit both activities,  $1 \mu\text{M}$  calmodulin protects against loss of cyclase activity but not against loss of invasiveness. Antibodies have been prepared that differentially inhibit the two activities by using as antigen either invasive or noninvasive bacterial cyclase. Polycations, such as polylysine, are more effective inhibitors of penetration than of cyclase activity. The effect on cyclase activity is biphasic, showing both stimulation and inhibition, and a polylysine concentration giving half-maximal inhibition of invasiveness caused half-maximal stimulation of cyclase activity. (Wolff, Raptis, Gentile).

#### IV. Interaction of Proteins with Cell Membranes

This section reports the concluding experiments in the study of clathrin and coated vesicles that were initiated by Dr. Harold Edelhoch. The interaction of clathrin-associated proteins (APs) with clathrin were examined under a variety of conditions. The ratio of APs to clathrin was found to govern the size of the resulting baskets; the lower the ratio, the lower the size. Above pH 6.5, however, only small-size baskets (150S) were formed, and clathrin associated stoichiometrically with the APs. Both 300S and 250S baskets dissociated into 150S baskets and free clathrin when the pH was increased from 6.0 to 6.5. It is probable that the 150S species represents an intermediate in basket formation, which requires the presence of APs. Electron microscopic examination suggests that clathrin may react with APs in smaller baskets and polymerize on their surface. Preformed baskets and isolated coated vesicles also were shown to interact with APs to form heavier species. Thus, the growth of basket structure may be analogous to microtubule formation, where microtubule subunits can attach to microtubule fragments when they are decorated with polycations such as DEAE or dextran. (Prasad, Lippoldt).

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 45000-22 CEB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Thyroxine-Protein Interactions

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. Robbins	Chief CEB, NIDDK
Others:	S. Benvenaga	Guest Researcher CEB, NIDDK
	H.J. Cahnmann	Scientist Emeritus CEB, NIDDK

## COOPERATING UNITS (if any)

University of Messina, Italy (S. Benvenaga).  
Dr. R.E. Gregg, Division of Intramural Research, NHLBI

## LAB/BRANCH

Clinical Endocrinology Branch

## SECTION

Hormone Metabolism and Action Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.3

## PROFESSIONAL:

2.3

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Since a portion of the thyroid hormones in plasma is bound to lipoproteins, we explored the possibility that these interactions may have a special role in the mechanism by which the hormones enter their target cells. LDL, which binds thyroxine (T<sub>4</sub>) through a specific interaction with apo B-100, was tested with normal human fibroblasts and fibroblasts deficient in LDL receptors. Both types of cells were shown to possess the usual stereospecific, energy-dependent uptake system for T<sub>4</sub> and T<sub>3</sub> (3,5,3'-triiodothyronine). In addition, in LDL receptor-competent fibroblasts, LDL increased the rate of uptake of T<sub>4</sub> in proportion to the amount of T<sub>4</sub> bound to LD. This presumably was mediated by receptor-mediated endocytosis of the LDL-T<sub>4</sub> complex. Since virtually all cell types express LDL receptors, the increased T<sub>4</sub> entry promoted by LDL should be of general significance.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 45004-18 CEB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structure of Polypeptide and Protein Hormones

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: K. Prasad Visiting Associate, CEB, NIDDK

Others: R. E. Lippoldt Health Services Ofcr., CEB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Clinical Endocrinology Branch

## SECTION

Protein Structure Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

.2

## PROFESSIONAL:

.2

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard un-reduced type. Do not exceed the space provided.)

During the last few years our laboratory has been interested in the structural characterization of clathrin and clathrin coated vesicles as they are related to receptor-mediated endocytosis. As the final work of this section we have made a study correlating the formation of the various sized clathrin basket structures with respect to the ratio of bound associated proteins to clathrin and to the polymerizing conditions.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 45009-22 CEB

PERIOD COVERED  
October 1, 1988 to September 30, 1989TITLE OF PROJECT (No characters or less. Title must fit on one line between the borders.)  
Studies in Thyroid Diseases

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. Robbins Chief, Clinical Endocrinology Branch CEB, NIDDK

Others: K. Ain Medical Staff Fellow CEB, NIDDK  
M. Phyllaier Biologist CEB, NIDDK

## COOPERATING UNITS (If any)

Dr. J. Norton, Surgery Branch, NCI; Dr. J. Reynolds, Nuclear Medicine, CC; M. Merino, Laboratory of Pathology, NCI; K. Denicoff, Biol Psychiatry Branch, NIMH; R. Polinsky, Clin Neuroscience Branch, NINDS

## LAB/BRANCH

Clinical Endocrinology Branch

## SECTION

Hormone Metabolism and Action Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.8

## PROFESSIONAL:

1.3

## OTHER:

.5

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects☐ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

Iodide and lithium cation both inhibit secretion of thyroid hormone and their effect is enhanced in a damaged thyroid gland. Since iodide is known to control hyperthyroidism after partial destruction by I131, we tested whether this was also true for lithium. It was shown that both iodide and lithium were effective, that the response to iodide exceeded that to lithium and that the 2 agents are additive in their effects.

In patients undergoing change in thyroid hormone status during routine radioiodine tests for thyroid cancer, some effects of short term hypothyroidism were tested. Hypothyroidism produced a measurable change in mood (increased sadness and anxiety), a decrease in systolic blood pressure and an increase in plasma norepinephrine. No difference in mood was detected when patients were maintained on T4 compared to T3.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 45014-18 CEB

PERIOD COVERED  
October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Membranes and Secretion

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J. Wolff Associate Chief CEB, NIDDK  
Others: D. L. Sackett Senior Staff Fellow CEB, NIDDK  
T. M. Shiver Medical Staff Fellow, CEB, NIDDK

COOPERATING UNITS (If any)

LAB/BRANCH  
Clinical Endocrinology Branch

SECTION  
Endocrine Biochemistry Section

INSTITUTE AND LOCATION  
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 2.0	PROFESSIONAL: 2.0	OTHER:
-------------------------	----------------------	--------

CHECK APPROPRIATE BOX(ES)  
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Attempts to understand the mechanism of microtubule involvement in steroidogenesis have led us to investigate lipid-tubulin interactions. Cholesterol binds to tubulin and the nature of this interaction is under study by both chemical and physical means.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 45016-19 CEB

PERIOD COVERED  
October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Thyroid Hormone Secretion and the Function of Microtubules

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. Wolff	Associate Chief	CEB, NIDDK
Others:	D.L. Sackett	Senior Staff Fellow	CEB, NIDDK
	R.E. Lippoldt	Chemist	CEB, NIDDK
	D. Zimmerman	Guest Worker (2 mos)	CEB, NIDDK
	G. Palumbo	Visiting Scientist (3 mos)	CEB, NIDDK

COOPERATING UNITS (if any)

Jay Knudsen, NHLBI; C. Gibson, DRS; Marc Lewis, DRS

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Endocrine Biochemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.0

PROFESSIONAL:

1.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The  $\alpha$  and  $\beta$  subunits of tubulin are not covalently bound despite the fact that one normally encounters only the dimer under "native" conditions. We have studied the interaction and equilibrium between the two subunits by three different methods: 1) rates of C-terminal proteolysis with subtilisin when the dimer is associated or dissociated; 2) binding of the fluorescent dye, Nile red, one site for which appears to be at the  $\alpha$ - $\beta$  contact site; 3) equilibrium centrifugation at very short column heights. All methods yield  $K_d$  values between  $10^{-7}$  and  $10^{-6}$ M. The basis for small differences is being studied, as are factors influencing the equilibrium.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 45018-14 CEB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Adenylate Cyclase and Other Extracellular Products of B. Pertussis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. Wolff	Associate Chief CEB, NIDDK
Others:	L. Knipling	Technician CEB, NIDDK
	A. Raptis	Visiting Fellow (9 mos)
	F. Gentile	Visiting Fellow

## COOPERATING UNITS (if any)

Organisms grown in P-3 facility of Bureau of Biologies, FDA through the kindness of C. R. Manclark and staff.

## LAB/BRANCH

Clinical Endocrinology Branch

## SECTION

Endocrine Biochemistry Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

3.2

## PROFESSIONAL:

2.2

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

- |   |  |   |
|---|--|---|
| <input type="checkbox"/> (a) Human subjects | <input type="checkbox"/> (b) Human tissues | <input checked="" type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors        |  |   |
| <input type="checkbox"/> (a2) Interviews    |  |   |

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Penetration of the invasive (or toxic) adenylate cyclase of Bordetella pertussis is shown to be charge dependent and can be inhibited by polycations of appropriate length. The effect on invasiveness is an order more sensitive than on inhibition of catalytic activity. Treatment of urea extracts containing invasive cyclase of B. pertussis with trypsin, chymotrypsin or subtilisin abolishes the ability to increase intracellular cAMP levels in CHO cells (Invasiveness) at concentrations that have minimal or no effects on adenylate cyclase activity. Higher protease concentrations can inhibit catalytic activity, and 1  $\mu$ M calmodulin protects this catalytic activity, but not invasiveness, against proteolytic inhibition. Rabbit IgG fractions from antisera prepared against urea extracts inhibited invasiveness at 10 fold lower concentrations than they inhibited catalytic activity. One IgG from rabbit immunized against a partially purified, non-invasive form of the B. pertussis adenylate cyclase inhibited catalytic activity but was ineffective against invasiveness. We have recently succeeded in obtaining highly purified forms of this very labile invasive cyclase and expect to gain substantial new insight to the domain structure of the toxin.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 45028-11 CEB

PERIOD COVERED  
October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Thyroid Hormone-Cell Interactions

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. Robbins	Chief CEB, NIDDK
	M. Lakshmanan	Medical Staff Fellow CEB, NIDDK
	E. Goncalves	Visiting Fellow CEB, NIDDK
	A. DiCerbo	Special Volunteer, CEB, NIDDK
	M. Phyllaier	Technician, CEB, NIDDK

COOPERATING UNITS (If any)

University Rio Grande du Sul, Porto Alegre, Brazil (Goncalves);

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Hormone Metabolism and Action Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

.8

PROFESSIONAL:

.3

OTHER:

.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

An increasing body of evidence suggests that triiodothyronine (T3) is transported through a carrier mediated energy dependent system into many cells, including rat myoblasts. However, conflicting data have been reported concerning the existence of a comparable system for the uptake of thyroxine (T4). In last year's report, we described active T4 uptake into central nervous system cells (neuroblastoma and glioma). In the present study we investigated how T4 is transported into rat myoblasts and compared its uptake process with that of T3. T4 uptake was about half that of T3 and could not be saturated by excess of T4 or T3. Labeled L-T4 and D-T4 were transported into myoblasts at similar rates. Preloading the cells with a high concentration of T4 did not alter the rate, in keeping with a diffusive process, and inhibitors of ATP production and of endocytosis had no effect. Thus T3 and T4 enter myoblasts by different mechanisms.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 45033-06 CEB

PERIOD COVERED  
October 1, 1988 to September 30, 1989TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Mapping of Triiodothyronine Responsive Genes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	V. M. Nikodem	Visiting Scientist	CEB, NIDDK
Others:	K. Petty	Medical Staff Fellow	CEB, NIDDK
	T. Mitsuhashi	Visiting Fellow	CEB, NIDDK
	B. Desvergne	Visiting Fellow	CEB, NIDDK
	M. Phyllaier	Biologist	CEB, NIDDK
	R. Lippoldt	Chemist	CEB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Hormone Metabolism and Action Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.9

PROFESSIONAL:

2.2

OTHER:

.7

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Triiodothyronine regulates the expression of several genes at the transcriptional level by mechanisms that are not adequately understood. As a model for examining triiodothyronine regulation of transcription, we used the promoter of a triiodothyronine-responsive gene, rat malic enzyme. To define the cis-regulating elements of this promoter, deletion mutants were transcribed in HeLa extracts in vitro. Three essential cis-regulatory elements were localized to -144/-123 (relative to the cap site at +1) containing a consensus binding site for the transcription factor AP-1, -70/-50 containing a unique 10 base pair direct repeat, and -30/+7 containing the cap site itself. The role of triiodothyronine in regulating these trans-acting factors was evaluated by gel shift assays using malic enzyme promoter oligonucleotide probes and rat tissue nuclear extracts. Results indicate that quantitative binding of liver nuclear proteins to two malic enzyme cis-regulatory elements (-144/-123 and -70/-50) was directly proportional to the thyroidal status of the animal. Furthermore, changes in the magnitude and time course of this promoter correlated closely with triiodothyronine induced increases in the malic enzyme gene transcription rate.

Gel shift assays with the malic enzyme genomic cDNA fragments (+33/-177 and -178/-354) showed that both in vitro translated receptors ( $\alpha$ ,  $\beta$ ) can complex only with the fragment -178/-354. This region in sequence with the malic enzyme promoter and acetyl chloramphenicol transferase can increase expression of this chimeric gene only in the presence of either receptor and thyroid hormone when transiently transfected in NIH 3T3 or COS7 cell lines. Detailed analysis of these sequences are underway.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 45034-06 CEB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Specific Rat Liver mRNAs by Thyroid Hormone

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	V. M. Nikodem, Ph.D.	CEB, NIDDK
Others:	D. Grieco, M.D.	CEB, NIDDK
	M. H. Song	CEB, NIDDK
	J.E. Rall	CEB, NIDDK
	R.E. Lippoldt	CEB, NIDDK

## COOPERATING UNITS (if any)

Dr. S.M. Aloj and Dr. L.Kohn, LBM, NIDDK

## LAB/BRANCH

Clinical Endocrinology Branch

## SECTION

Hormone Metabolism and Action Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

.4

## PROFESSIONAL:

.1

## OTHER:

.3

## CHECK APPROPRIATE BOX(ES)

- |   |  |   |
|---|--|---|
| <input type="checkbox"/> (a) Human subjects | <input type="checkbox"/> (b) Human tissues | <input checked="" type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors        |  |   |
| <input type="checkbox"/> (a2) Interviews    |  |   |

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The availability of cDNA and genomic sequences for rat malic enzyme allowed us to probe for the molecular mechanism by which dehydroepiandrosterone increases the malic enzyme activity.

Dehydroepiandrosterone, a naturally occurring steroid secreted from the adrenal, has been reported to decrease the body weight gain in rodents without suppressing food intake, and to stimulate malic enzyme activity in liver. In this report, we demonstrate that dehydroepiandrosterone induces hepatic malic enzyme activity by increasing the rate of transcription of the malic enzyme gene. This transcriptional activation of the malic enzyme gene is dose dependent, i.e., the treatment of euthyroid male rats with daily doses of 17.5 mg and 35 mg dehydroepiandrosterone per 100g body weight for 7 days elevated the rate of malic enzyme gene transcription in liver 4-5 and 8-9 fold, respectively, above the basal levels. The levels of nuclear malic enzyme RNA, cytoplasmic malic enzyme mRNA, and enzyme activity were increased correspondingly. Malic enzyme stimulation by dehydroepiandrosterone was liver-specific, i.e., malic enzyme activity in brain, heart, kidney, and testis was unchanged. Thyroid hormone is required for the induction of hepatic malic enzyme activity by dehydroepiandrosterone since in hypothyroid animals dehydroepiandrosterone was without effect. However, malic enzyme stimulating effects of T3 and dehydroepiandrosterone are additive in euthyroid rat livers at both levels of gene transcription and enzyme activity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 45037-04 CEB

PERIOD COVERED  
October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Hormones and Cell Differentiation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Pontecorvi Visiting Fellow CEB, NIDDK  
Others: M. Phyillaier Biologist CEB, NIDDK  
J. Robbins Chief CEB, NIDDK  
J. Tata Fogarty Scholar, CEB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Clinical Endocrinology Branch

SECTION

Hormone Metabolism and Action Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been discontinued.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 45038-02 CEB

PERIOD COVERED  
October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Molecular biology of thyroid hormone receptors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	V.M. Nikodem	Visiting Scientist	CEB, NIDDK
Others:	T. Mitsuhashi	Visiting Fellow	CEB, NIDDK
	M. Phyllaier	Biologist	CEB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH  
Clinical Endocrinology Branch

SECTION  
Hormone Metabolism and Action Section

INSTITUTE AND LOCATION  
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 1.1	PROFESSIONAL: 1.1	OTHER:
-------------------------	----------------------	--------

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

The rat  $\alpha$ -thyroid hormone receptor gene encodes through alternative splicing at least three protein isoforms with different functions, and three mRNA species (2.6, 5.4, 6.8 kilobase (kb) in size) are detected using  $\alpha$  gene-specific probes. In the present study, the identities of these mRNAs were analyzed by Northern analysis, and it was demonstrated that in rat brain the receptor protein is encoded by the minor 5.4- and 6.8-kb mRNAs and the variant proteins are encoded by the major 2.6-kb mRNA. Relative quantities of these mRNAs were determined by RNase protection assay, and the ratio of the receptor mRNAs to the variant mRNAs was estimated to be 1:6 in adult brain. The ratio between the mRNAs was regulated in both a tissue-specific and developmental stage-specific manner. The receptor mRNA levels were also regulated by the thyroid state of the animal showing an increased level in hypothyroid rat liver while those in brain were not affected. Analysis of the  $\alpha$ -thyroid hormone receptor gene suggested that the choice between two polyadenylation sites and subsequent RNA processing appear to generate the 3' heterogeneity of alternative mRNAs.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 45040-01 CEB

PERIOD COVERED  
October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders)  
Effect of thyroid hormone on synthesis of myelin basic proteins

PRINCIPAL INVESTIGATOR (Use other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Farsetti Visiting Fellow, CEB, NIDDK  
Others: V. Nikodem Senior Investigator, CEB, NIDDK  
T. Mitsuhashi Visiting Associate, CEB, NIDDK  
J. Robbins Chief, CEB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH  
Clinical Endocrinology Branch

SECTION  
Hormone Metabolism and Action Section

INSTITUTE AND LOCATION  
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 1.3	PROFESSIONAL: 1.3	OTHER:
-------------------------	----------------------	--------

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Fetal and neonatal thyroid status affects the growth, development and maturation of the mammalian central nervous system. These processes are dependent on the presence of thyroid hormones during the so-called "critical period". During this period of development, active myelination takes place. In mice, MBP is encoded by a single gene. The primary transcripts are alternatively spliced to generate mRNAs for four proteins of molecular weight  $\approx$  14, 17, 18.5 and 21 kDa. The purpose of this study was to analyze and quantitatively evaluate all MBP mRNA isoforms during mouse brain development and assess the effect of hypothyroidism on their expression in the developing and adult brain. Dot blot hybridization analyses revealed that MBP RNA expression can be detected as early as 2 days after birth in the mouse with maximal expression occurring between 16 and 20 days post-natally followed by a decrease in the adult mouse. Slot blot and RNase protection assay of RNA prepared from brain of adult hypothyroid mice showed that the concentrations of MBP mRNAs are about 5 fold lower when the levels of these RNAs are compared with those found in euthyroid mice. Thus it appears that the thyroidal status can alter expression of this gene even in adult brain despite the belief that the mature brain is refractory to thyroid hormones. To investigate the molecular basis of effects of thyroid hormone on expression of MBP, a "run on" assay will be used to determine changes in transcription rates of this gene as a result of thyroid hormone action.

## ANNUAL REPORT OF THE DIABETES BRANCH

National Institute of Diabetes and Digestive and Kidney Diseases

### Introduction

The Diabetes Branch continues to pursue a broad based program which encompasses clinical research, studies on the mechanism of insulin action, with special emphasis on the nature and function of the insulin receptor, studies on the evolution of hormones and their function as messenger molecules, gene sequencing of insulin and insulin-like growth factors I and II, studies on morphological interaction of hormones with cells, and detailed studies of the biosynthesis of the insulin receptor.

### Recognition of Previous Achievements

Several grants were awarded to members of the Diabetes Branch: Juvenile Diabetes Foundation; American Diabetes Foundation, both the national organization and local affiliate; Diabetes Foundation, the Upjohn Company.

A publication by Dr. L. Bassas, Dr. F. De Pablo, Maxine A. Lesniak and Dr. J. Roth received the Serono Award for "Research about growth problems". Dr. Martin Adamo received a JDF Research Grant in 1989. Dr. Charles T. Roberts, Jr. a ADA Research Grant and also a Diabetes Research and Education Foundation Research Grant in 1989. Dr. Derek LeRoith co-chaired International Symposium on Molecular Biology of Insulin and IGF's and their Receptors in Gainesville, Florida in 1989. Dr. LeRoith also was Invited Lecturer at Warren F. Wilhelm Lectureship, Ed Bixby Research Institute, Kansas City, MO. Dr. Jesse Roth is continuing as a Board Chairman of the Weizmann Institute in Israel. In February 1988 he was Organizing Chairman, at the Annual Meeting of Italy-NIH Collaborative Research Program in Diabetes. In March 1988, Dr. Roth was Work Group Chairman, Second World Conference on Diabetes, Juvenile Diabetes Foundation, Monaco; Dr. Roth was also Course Organizer, Molecular and Cellular Biology of Intercellular Communication, Foundation for Advanced Education in the Sciences, NIH. Dr. Roth received the (First Annual) National Medical Research Award, National Health Council; the Steven C. Beerring Award at the University of Indiana. Dr. Jesse Roth received an honorary degree from the University of Rome, Rome, Italy in April 1989.

## Receptors for Insulin and Insulin-like Growth Factor I (IGF-I)

### Characterization of the receptors

Both the insulin and IGF-I receptors are heterotetrameric glycoproteins with two identical alpha subunits and two identical beta subunits joined by disulfide bridges. The alpha subunit (135kDa) which lies entirely extracellularly is the hormone binding subunit of the receptor and the beta-subunit (9kDa) which spans the cell membrane is a tyrosine kinase capable of phosphorylating itself as well as a number of specific endogenous and exogenous substrates.

Biosynthetic labeling of the insulin receptor was performed with radio-active sugars and tryptic peptides were separated using HPLC. Specific glycosidases have been employed to demonstrate that the insulin receptor contains O-linked oligosaccharides in addition to the previously described N-linked oligosaccharides. The O-linked carbohydrates are on the beta-subunit. The functional significance of this as well as other post-translational modifications of the insulin receptor i.e. fatty acylation and phosphorylation is being studied. These changes may be important in intracellular processing of the receptor or in functional aspects of the receptor while it is on the cell surface. Thus, the proreceptor precursor (190kDa), which contains one alpha and one beta subunit is capable of forming dimers, of phosphorylation and activation of tyrosine kinase activity. Thus, dimerization may be another modification that is important for activity.

In an ongoing collaboration with the group in Geneva, Switzerland, it was shown by electron microscopy that in cells derived from patients with insulin resistance, insulin associated more with the non-villous portion of the cell surface which suggests the presence of fewer villous portions and may be a factor related to insulin resistance. In addition, internalization of insulin and its receptor, an early event in insulin action, was decreased in the face of hyperglycemia.

### Antibodies to the insulin receptor

The usefulness of antibodies to the insulin receptor in understanding insulin action is being pursued by the Diabetes Branch. Autoantibodies to the receptor in patients with insulin resistance are being studied and one such group of antibodies has been shown to immunoprecipitate the receptor without interfering with insulin binding. Further studies have included the production of antibodies in rabbits to specific sequences of the insulin receptor. These antibodies will prove useful in delineating different sequence motifs, and thereby further our understanding of insulin resistance states.

### Tissue specific forms of the receptor

Insulin and IGF-I receptors are widespread throughout the body being expressed by most tissues and cells including the central nervous system. In preparations of whole brain these receptors have alpha subunits that are about 10 kDa smaller than those from peripheral tissues due to differences in glycosylation. In primary cell culture, glial and neuronal cells express both insulin and IGF-I receptors. The neuronal cell receptors are of the "brain type" whereas the glial receptors are of the larger peripheral-type. The relationship of these structural changes to functional aspects was tested in these cells. Insulin stimulated glucose uptake in glial cells similar to its effect on classic target cells whereas it had no effect on neuronal cell glucose uptake.

### Receptor tyrosine kinase and specific endogenous substrates

Ligand-induced autophosphorylation of the insulin and IGF-I receptors, is considered to be an important early step in the action of these hormones. This is followed by the phosphorylation of endogenous substrates especially on tyrosine (but also on serine) residues. In rat liver a 120 kDa glycoprotein (ppl20) can be phosphorylated by solubilized insulin, IGF-I and EGF receptors. Using a monoclonal antibody (HA<sub>4</sub>) ppl20 has been immuno-affinity purified and partial amino acid sequence of the protein obtained. The sequence has allowed the generation of oligonucleotide probes for the purpose of isolating cDNAs encoding ppl20.

The phosphorylation of insulin and IGF-I receptors was increased in both neuronal and glial cells suggesting that these receptors undergo similar changes compared with their non-neural counterparts, following ligand binding. In neuroblastoma cell lines an endogenous substrate ppl85 (phosphoprotein 185 kDa) was phosphorylated on tyrosine residues following insulin and IGF-I stimulation. The relationship of this endogenous substrate to insulin and IGF-I action in these cells will require purification of the protein and cloning of the corresponding cDNA.

Studies in patients with type II diabetes have been performed and revealed a generalized defect in the relationship of the alpha and beta subunit functions. Thus, the amount of insulin-induced tyrosine kinase activity per unit of insulin binding is decreased. These data suggest a defect in the alpha subunit coupling to beta subunit in signal transduction or alternatively an abnormality in tyrosine kinase itself. To pursue these question, a patient with selective tyrosine kinase defect and genetic type A insulin resistance is being studied in an attempt to identify the specific molecular defect.

### Receptors for insulin and insulin-like growth factors in brain and embryo.

Specific insulin receptors are widely distributed throughout the rat brain. Using autoradiographic techniques, specific insulin as well as IGF-I and IGF-II receptors were visualized in many areas of the brain, particularly in the choroid plexus, olfactory bulb, limbic areas and cerebellum. The concentration of these receptors was measured in two models: 1. streptozotocin-induced diabetes in the rat and 2. the purkinje cell deficient (pcd) mouse. In both models the concentration of insulin, IGF-I and IGF-II receptors was unchanged. Thus, these receptors were unaffected by the severe metabolic derangement associated with streptozotocin-induced diabetes nor were they related to the

neuronal degeneration seen in pcd mice.

The role of insulin and related growth factors in embryogenesis is being studied in the chicken embryo. Both insulin and IGF-I receptors are demonstrable as early as organogenesis is recognized. Precise localization of these receptors in developing chicken embryo brains was done and compared to the pattern of adult brain. Whereas in young embryos both hormones showed very similar localization of binding, in late embryos and adult brain the insulin and IGF-I binding patterns were distinct. These differences may be useful in studies related to the role of each hormone in embryogenesis.

#### Studies of Human Disease

##### Molecular defects in syndromes of extreme insulin resistance

One of the main areas of research in the Diabetes Branch has centered on studying the role of insulin resistance in diabetic states especially type II diabetes. As part of this ongoing project, a number of syndromes with very severe insulin resistance have been studied. These include genetic forms of insulin resistance e.g. leprechaunism and type A insulin resistance.

With the aid of the normal human insulin receptor cDNA, the molecular defects in the cells of these patients have been studied. Our family in which two sisters have a genetic form of insulin resistant diabetes mellitus (type A) The technique of homozygosity mapping showed that the mutation causing diabetes in the causangimous family is linked to the insulin receptor gene. The two insulin resistant sisters are homozygous for a mutation encoding a substitution of valine for phenylalanine at position 382 in the alpha-subunit, which in transfection experiments caused an impairment of processing of the receptor and resulted in lower levels of insulin binding to cells. This defect could then explain the insulin resistance in these patients. Cells from a second patient, with extreme insulin resistance, from Japan, were found to have a mutation in which valine is substituted for glycine 996 in the beta subunit of the insulin receptor. This glycine normally forms an important part of the putative ATP binding site and the mutation almost certainly explains the decrease tyrosine kinase activity of the insulin receptor and probably also the insulin resistance found in this patient.

##### Positron emission tomography (PET) in patients with diabetes mellitus:

Insulin was found to increase the rate of loss of intracellular 2 deoxy-glucose- $PO_4$  from grey matter both in normals and obese Pima Indians with or without diabetes. The effect was most pronounced in the premotor and pre-frontal brain regions.

##### Studies of other related disease states.

The diabetes Branch has long been interested in diseases, other than diabetes, associated with abnormal carbohydrate metabolism of Acromegaly and hypoglycemia produced by insulinomas. The new long-acting analogue of somatostatin, SMS 201-995 has been used experimentally in these patients to control the symptoms associated with these hormone producing tumors. SMS 201-995 has been found to be effective in suppressing circulating hormone levels in TSH-producing tumors and GH-producing pituitary adenomas with some clinical improvement in a sub-group of patients.

Acromegalic patients have been followed for many years and it appears that joint disease which is associated with acromegaly is a function of age of the patient and/or the degree of involvement at initial therapy.

### Insulin and IGF-I Gene Expression

With the advent of molecular biology it is now easy to determine the expression of many different genes and the regulation of this expression in developmental stages as well as in disease states. Thus, since the expression of insulin and IGF-I are important in normal development and differentiation, the Diabetes Branch has become involved in these types of studies.

The ontogeny of expression of the insulin gene has been studied in the chicken embryo and the effect of insulin and IGF-I on chicken lens development has been studied. Insulin gene expression begins early in embryogenesis, even before pancreatic development. Furthermore, insulin and especially IGF-I significantly increase gene transcription of delta-crystallin in the lens epithelial and fiber cells.

In another model of development *Xenopus laevis*, cDNAs for insulin have been cloned and sequenced. These studies have demonstrated that *Xenopus* have two non-allelic insulin genes. These cDNAs can now be used to study insulin gene expression during embryogenesis as well as to perform studies to determine insulin role in *Xenopus* embryogenesis.

A transgenic mouse line, previously established, is being characterized to determine the effect of overexpression of insulin. Indeed, these mice seem to represent an excellent model for the study of type II diabetes.

The role of IGF-I in disease states is being studied from many aspects. Both the rat IGF-I and the IGF-I receptor cDNAs have been cloned and sequenced and further studies include the search for their respective promoters to determine the regulation of gene expression in different disease states. Developmental studies in the rat have determined that IGF-I is minimally expressed at neonatal stages and progressively increased towards puberty and adult life. In contrast, IGF-I receptor gene expression is maximum in most tissues around birth and declines postnatally. These studies point to the importance of the IGF-I receptor in early development and suggest that IGF-I itself may be more important in later stages. In both fasting and streptozotocin-induced diabetes, the expression of IGF-I in the liver is decreased; refeeding or insulin therapy in the diabetic animals restores gene expression. These findings may explain the stunted growth found in malnourished children with or without diabetes.

To further explore the regulation of IGF-I gene expression we have identified a number of splicing variants in the IGF-I mRNAs that may be directly related to translational control of IGF-I. The variant predominantly found in liver called class A, has an inverted repeat in its 5' and 3'-untranslated regions. Folding of the molecule with annealing of these inverted repeats may stabilize the molecule and prevent translation. This regulation of translation may be important in control of IGF-I production in liver and may be affected by various disease states including growth retardation, malnutrition and diabetes.

### Unification Hypothesis

Systems of intercellular communication in mammals include endocrine, neurocrine and paracrine, and others. These systems bear striking similarities to each other especially in regard to their biochemical elements. In addition, the signal molecules used as messengers in these systems are also found in non-vertebrate systems and have led to the suggestion of an earlier evolutionary origin than has previously been appreciated as well as a wider biological role for these systems of intercellular communication. In addition to demonstrating the presence of material resembling mammalian insulin in unicellular organisms and higher plants we and others have also found other hormone-like molecules e.g. ACTH and somatostatin in these diverse organisms. As part of the characterization of these materials we have continued to purify them using sophisticated chromatographic analysis e.g. high performance liquid chromatography, affinity chromatography. Once the peptide(s) are purified to homogeneity, each will be subjected to amino acid composition and sequence analysis. The sequence analysis information will be used to generate appropriate synthetic oligonucleotides to enable us to screen the genomes of these unicellular organisms to isolate and identify the genes encoding these hormone-like peptides. These studies will eventually enable us to understand the function of the peptides in these organisms and by extrapolation to identify other possible functions of these peptides in mammals.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 47001-08 DB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Phosphorylation of the Insulin and IGF-I Receptors

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I.	D. LeRoith	Section Chief	DB/NIDDK
Others	M. Adamo	Guest Researcher	DB/NIDDK
	Z. Shen-Orr	Guest Researcher	DB/NIDDK
	H. Werner	Guest Researcher	DB/NIDDK
	M. Woloschak	Medical Staff Fellow	DB/NIDDK
	M. Bach	Medical Staff Fellow	DB/NIDDK

## COOPERATING UNITS (if any)

University of Florida, Gainesville, FL (M. Raizada)

## LAB/BRANCH

Diabetes Branch

## SECTION

Section of Molecular and Cellular Physiology

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

6.0

## PROFESSIONAL:

5.0

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Brain insulin receptor and insulin-like growth factor I (IGF-I) receptors are similar to their peripheral, non-neural counterparts, being comprised of two alpha subunits and two beta subunits in a heterotetrameric formation. However, they have smaller apparent Mr compared to the peripheral receptors on sodium dodecylsulfate-polyacrylamide gel electrophoresis.

These unique insulin and IGF-I receptors have been studied in membranes prepared from whole retina, brain, peripheral nerves, as well as from neural-derived cultured cells. Primary cultures of neuronal cells contain unique insulin and IGF-I receptors resembling those of whole brain. Peripheral nerves and glial cells on the other hand, contain insulin and IGF-I receptors similar to those found in non-neural tissues.

Insulin and IGF-I induce autophosphorylation of their respective receptors as well as endogenous substrates in intact cells in culture. In addition, both ligands stimulate gene expression of the rat brain/HepG2 glucose transporter probably resulting in an increase in constitutive glucose transport. Since insulin stimulates glucose uptake in glial and not neuronal cells, this suggests that a second separate glucose transporter may be involved as well.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 47002-02 DB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin Gene Expression and Insulin Action

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	F. De Pablo	Visiting Scientist			DB/NIDDK
	J. Roth	Director, DIR			DB/NIDDK
Others:	J. Serrano	Visiting Associate	L. Scavo	Guest Worker	DB/NIDDK
	A. Shuldiner	Senior Staff Fellow	L. Scott	IRTA	DB/NIDDK
	L. Marban	Guest Worker	A. Nirula	Spec. Vol.	DB/NIDDK
	J. Alemany	Visiting Fellow			DB/NIDDK
	R. Dashner	Microbiologist			DB/NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Diabetes Branch

## SECTION

Receptor and Hormone Action Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

8.7

## PROFESSIONAL:

5.7

## OTHER:

3

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Molecular biological techniques are being used to discern gene expression of insulin, insulin-like growth factors (IGFs) and their receptors and actions in several vertebrate model systems. Insulin is apparently a requirement for normal development, and the chicken embryo is one of the suitable models for studying the role of insulin in development. The ontogeny of expression of the insulin gene has been studied and in order to undertake the characterization of the expression of IGF-I, the gene for this factor is being cloned. The role of insulin in cell differentiation and gene expression in the eye lens of chick embryo is also being used as a cell model to understand the action of insulin in development. The amphibian, Xenopus laevis, is also a model system used to study development. Previously the amphibian insulin sequence was unknown. In this laboratory insulin from pancreas of Xenopus has been isolated and its sequence characterized and confirmed by molecular cloning. Studies are in progress to define the expression of insulin during Xenopus development using the polymerase chain reaction.

A transgenic mouse line with multiple copies of the human insulin gene integrated into its genome has been established. The degree of hyperinsulinemia correlates with human gene copy numbers. The transgenic mice provide a model system for studies in regulation of insulin gene expression and the effects of chronic hyperinsulinemia on glucose homeostasis.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 47005-17 DB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Studies of insulin receptors in circulating cells in man		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.	P. Gorden	Director  NIDDK
Others:	S.I. Taylor C. Hendricks R. Arakaki O. Rodriguez	Section Chief, Biol. Lab Tech. Senior Staff Fellow Special Volunteer  DB/NIDDK DB/NIDDK DB, NIDDK DB, NIDDK
COOPERATING UNITS (if any) Wayne State University (G. Grunberger)		
LAB/BRANCH Diabetes Branch		
SECTION Clinical and Cellular Biology Section; Biochemistry and Molecular Pathology Section		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>The present work continues prior investigations of <u>insulin receptors</u> on circulating cells in patients with <u>insulin resistance</u> and <u>diabetes mellitus</u>. The effects of diet, fasting and treatment on receptor function are under investigation. Insulin receptors are evaluated for their ability to bind insulin and to act as tyrosine-specific protein kinases.</p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER  Z01 DK 47007-14 DB
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PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

**Antibodies to Receptors: Detection in Disease States and Use as Probes**

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	Simeon Taylor	Chief, Biochem. & Molec. Path. Section,	DB,NIDDK
Others:	Domenico Accili	Special Volunteer	DB,NIDDK
	Victoria Moncada	Summer Student (Biologist)	DB,NIDDK
	Bernice Samuels,	Chemist	DB,NIDDK
	Fabrizio Barbetti	Visiting Fellow	DB,NIDDK
	Carla Hendricks	Biotech.	DB,NIDDK
	Jesse Roth	Scientific Director	NIDDK
	Phillip Gorden	Director	NIDDK

COOPERATING UNITS (if any)

Sao Paulo, Brazil (Walter Bloise)  
Sao Paulo, Brazil (Bernardo L. Wajchenberg)

LAB/BRANCH

**Diabetes Branch**

SECTION

**Biochemistry & Molecular Pathogenesis Section**

INSTITUTE AND LOCATION

**NIDDK/NIH/Bethesda, Maryland**

TOTAL MAN-YEARS:

4.5

PROFESSIONAL:

2.0

OTHER:

2.5

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Antibodies directed against the insulin receptor have played a central role in investigations of the insulin receptor structure and function. Initially, these antibodies were identified in the serum of patients with autoimmune forms of extreme insulin resistance or hypoglycemia. All of the anti-insulin receptor autoantibodies in the original studies shared the ability to inhibit insulin binding. More recently, however, we have identified a patient whose serum contained anti-receptor antibodies which immunoprecipitated the insulin receptor without inhibiting insulin binding.

In addition, based on the recently elucidated primary sequence of amino acids in the human insulin receptor, we have synthesized peptides corresponding to specific structural domains in the receptor. Rabbits have been immunized with these peptides in order to develop anti-receptor antibodies directed against specific site in the receptor. The antibodies have been used to identify structural abnormalities in patients with insulin resistant diabetes.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 47009-02 DB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Positron Emission Tomography in Patients with Diabetes Mellitus

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. J. Roth Director, DIR NIDDK

Others: R. Eastman Clinical Director DB/NIDDK

## COOPERATING UNITS (if any)

D. Bogardus, Phoenix, AZ  
K. Baker, R.N. CC, A. Cassibry, RN, CC  
G. Berg, MSF Nucler Medicine

## LAB/BRANCH

Diabetes Branch

## SECTION

Receptor and Hormone Action Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland

## TOTAL MAN-YEARS:

2

## PROFESSIONAL:

2

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Brain glucose metabolism was studied in Pima Indians using positron emission tomography (PET) under basal conditions and under hyperinsulinemic (1600 microunits/ml), euglycemic conditions to determine whether brain glucose metabolism is normal in non-insulin-dependent diabetes mellitus, and whether insulin has a regional affect on brain glucose metabolism in man.

Analysis of the data indicates that central nervous system glucose in Pima Indians without diabetes and with diabetes of 1-12 years duration is normal. Insulin was found to have no effect on glucose metabolism in any subregion of the brain analyzed. Insulin, however, was found to increase the rate (k4) of loss of intracellular 18F-fluoro-2-deoxy-glucose-6-P04 from grey matter. As measured by PET k4 is thought to represent the rate of hydrolysis of glucose-6-phosphate by glucose-6-phosphatase. We observed a 30-50% increase in k4 during the hyperinsulinemic studies. The effect was observed in both diabetic and non-diabetic Pima. The effect was most pronounced in the premotor and prefrontal brain regions.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 47014-20DB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Acromegaly and Growth Hormone

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	P. Gorden	Director	NIDDK
Others:	C. M. Hendricks	Bio. Lab. Tech.	DB, NIDDK
	R. F. Arakaki	Senior Staff Fellow	DB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Diabetes Branch

## SECTION

Clinical and Cellular Biology Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

1.0

## OTHER:

.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Acromegalic patients have continued to be followed with respect to pituitary irradiation. Further, we are evaluating the effects of transsphenoidal hypophysectomy in these patients and comparing them to the pituitary-irradiated patients.

A group of patients in a long-term follow-up study was evaluated to determine the effect of joint disease as a function of time following pituitary radiation. It appears that the joint disease is a function of the age of the patient and/or the degree of involvement at initial therapy. Thus in patients with relatively severe joint disease, the joint disease progresses in spite of very significant reductions in growth hormone following radiation treatment. Other studies underway are attempting to determine the possible effect of radiation on pituitary function or other complications of therapeutic maneuver.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 47018 12 DB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cellular Hormone-Like Peptides

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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## COOPERATING UNITS (if any)

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## LAB/BRANCH

Diabetes Branch

## SECTION

Molecular and Cellular Physiology Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

5.0

## PROFESSIONAL:

4.0

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Substances similar to insulin, ACTH and somatostatin are present in unicellular organisms and higher plants. The studies have been extended to further characterize the insulin, ACTH- and somatostatin-related molecules in spinach, E.coli and Saccharomyces. Using gel chromatography and high performance liquid chromatography the extracted materials have been purified in preparation for amino acid sequencing. Partial sequence analysis has been obtained and this information will be used to synthesize oligonucleotides to probe genomic E.coli libraries. Meanwhile antibodies are being used to screen these  $\lambda$ GTII expression libraries.

To assist in this work rat IGF-I cDNAs have been cloned. These cDNAs have also been used to study transcriptional and translational control of IGF-I gene expression.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 47019-12 DB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Morphologic studies of ligand binding to cells		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)  <div style="display: flex; justify-content: space-between;"> <span>P.I.</span> <span>P. Gorden</span> <span>Director</span> <span>NIDDK</span> </div>		
COOPERATING UNITS (if any)      Institute of Histology and Embryology, University of Geneva School of Medicine, Geneva, Switzerland. (J.L. Carpentier, A. Roberts, L. Orci) - Foreign		
LAB/BRANCH Diabetes Branch		
SECTION Clinical and Cellular Biology Section		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: <div style="text-align: center;">1.0</div>	PROFESSIONAL: <div style="text-align: center;">1.0</div>	OTHER:
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div> <input type="checkbox"/> (a) Human subjects              <input type="checkbox"/> (a1) Minors              <input type="checkbox"/> (a2) Interviews         </div> <div> <input checked="" type="checkbox"/> (b) Human tissues         </div> <div> <input type="checkbox"/> (c) Neither         </div> </div>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>             This work represents over 12 years of collaboration between the Diabetes Branch and the Institute of Histology and Embryology at the University of Geneva. The initial observations demonstrated that polypeptide hormones are taken up by the cell through a process of <u>receptor-mediated endocytosis</u> similar to other biologically important ligands that bind to cells. In the present study, using electron microscopy, we find a) there is anatomical correlation between the dissociation of <sup>125</sup>I-insulin and its localization on the cell surface. This work has now been extended to include an <u>insulin-resistant</u> cell line that has an abnormal surface which leads to a higher association of ligand to the non-villous portion of the cell surface. Further, receptor-mediated endocytosis also appears to be regulated in hypoinsulinemic states. In both rat and in human type I diabetes there is an inhibition of <sup>125</sup>I-insulin internalization in the hyperglycemic state, the normal state is restored by insulin treatment. The role of intracellular calcium on the endocytotic process as well as the relationship of stimulators of protein kinase C to internalization of both insulin and unrelated ligands such as <u>transferrin</u> have been studied also. In addition, the function of the small non-coated invaginations in receptor-mediated endocytosis is being investigated.           </p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 47022-10 DB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Insulin receptors in syndromes of extreme insulin resistance		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.	Simeon Taylor	Section Chief, DB, NIDDK
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	Takashi Kadowaki	Visting Fellow, Carla Hendricks Biotech. DB, NIDDK
	Hiroko Kadowaki	Special Volunteer, Jesse Roth Scientific Dir. DB, NIDDK
	Domenico Accili	Special Volunteer, Phillip Gorden Director DB, NIDDK
	Catherin Frapier	Secpial Vol. Amy Patterson Med. Staff Fel. DB, NIDDK
	Bernice Samuels	Chemist, Tony Pham Spec. Vol. DB, NIDDK
	Catherine McKeon	Senior Staff Fellow DB, NIDDK
COOPERATING UNITS (if any) Stephen Lilioja, Phoenix, AZ, NIDDK; Clifton Bogardus, Phoenix, AZ, NIDDK; Eric Lander, Whitehead Inst. & Harvard Univ., Cambridge, MA; Emilio Ramos, Univ. of MD, Alex Ullrich, Max Planck Inst. Munich, Germany; Luitgard Mosthaf, Max Planck Instit., Munich, Germany; Masato Kasuga, Tokyo Univ., Japan; Masato Odawara, Tokyo Univ., Tokyo, Japan; Adela Rovira, Madrid, Spain.		
LAB/BRANCH Diabetes Branch		
SECTION Biochemistry & Molecular Pathogenesis		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
10.0	8.0	2.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>Insulin resistance contributes to the pathogenesis of several obesity and noninsulin-dependent diabetes mellitus. We have investigated the insulin receptor gene in patients with genetic forms of insulin resistance to gain insight into biochemical defects which give rise to disease.</p> <p>Previously, we have identified a mutation in the insulin receptor gene of a patient with leprechaunism which encodes substitution of glutamic acid for lysine at position 460 in the alpha-subunit. To elucidate the mechanism whereby this mutation interferes with the function of the receptor, we have studied cells transfected with the mutant receptor cDNA. In addition, for comparison, we have constructed cDNA's encoding mutant receptors in which other amino acids are substituted for lysine at position 460. These studies have suggested that the patient's mutation interferes with the ability of acidic pH to strip the ligand off the receptor. This insensitivity to changes in pH appears to interfere with recycling of the internalized receptor, thereby accelerating the rate of receptor degradation and decreasing the number of insulin receptors on the cell surface.</p> <p>We have identified a mutation in the insulin receptor of two sisters with type A extreme insulin resistance. These patients are homozygous for a mutation (substitution of valine for phenylalanine at position 382 in the alpha-subunit which impairs the post-translational processing and transport of the receptor to the plasma membrane. This mutation appears to be recessive.</p> <p>We have identified another mutation in an insulin resistant Japanese man. This mutation encodes substitution of valine for glycine at position 996 in the beta-subunit, the third glycine in the Gly-X-Gly-X-X-Gly motif of the ATP binding site in the tyrosine kinase domain.</p> <p>At present we are amplifying and sequencing the coding sequences of insulin receptor genes from several insulin resistant patients. In addition, we cloned insulin receptor cDNA from an insulin-resistant Pima Indian with a strong family history of diabetes. Both of the alleles of this Pima Indian's insulin receptor gene encode <math>\beta</math>-subunits with normal deduced amino acid sequences. The remainder of the coding sequence is presently being determined.</p>		

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 47024-10DB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biosynthetic Labeling of the Insulin Receptor

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. E. Collier,

Expert

Others: R. F. Arakaki  
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P. GordenSenior Staff Fellow  
Visiting Associate  
Director

## COOPERATING UNITS (if any)

## LAB/BRANCH

Diabetes Branch

## SECTION

Clinical and Cellular Biology Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland

## TOTAL MAN-YEARS:

3.0

## PROFESSIONAL:

3.0

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have studied the post translational modifications of the insulin receptor, i.e., glycosylation, fatty acylation, and phosphorylation using biosynthetic labeling of the insulin receptor. After labeling the insulin receptor with radioactive sugars, the individual subunits were isolated and tryptic peptides were made from each subunit. These peptides were separated using High Performance Liquid Chromatography and the glycosylated peptides detected by counting the fractions in a liquid scintillation counter. Based on the differentially sensitivity of these labeled peptides to peptide N-glycosidase F, an enzyme that removes high mannose and complex N-linked oligosaccharides, and endo-a-N-acetylgalactosaminidase, an enzyme that removes O-linked oligosaccharides, we have shown that the insulin receptor contains O-linked oligosaccharides. These O-linked carbohydrates are on the beta subunit. In addition, we investigated the function of the insulin proreceptor by separating the precursor from the mature receptor by lectin chromatography. The insulin receptor precursor exhibited insulin stimulated endogenous and exogenous phosphorylation activity. It was also able to form dimers.

Regulation of insulin receptor gene expression by hydrocortisone and insulin was also investigated. Hydrocortisone but not insulin was shown to increase the rate of transcription of the insulin receptor gene.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 47025-06 DB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Tissue Receptors for Insulin and Insulin-like Growth Factors

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

P.I.	F. de Pablo	Visiting Scientist	DB/NIDDK
	J. Roth	Director, DIR	DB/NIDDK
	M.A. Lesniak	Chemist	DB/NIDDK
Others:	M. Rojeski	Senior Staff Fellow	DB/NIDDK

## COOPERATING UNITS (if any)

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## LAB/BRANCH

Diabetes Branch

## SECTION

Receptor and Hormone Action Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland

## TOTAL MAN-YEARS:

3.0

## PROFESSIONAL:

3.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Insulin receptor and insulin-like growth factors (I and II) receptors structure studies have been extended in rat brain. The binding of labeled peptides to thin sections of frozen fresh rat brain was visualized with autoradiography and computer-assisted densitometric analysis. By several criteria including structure-activity relationship analysis, these brain peptides receptors were qualitatively indistinguishable from peptide receptors previously characterized on brain and other more typical target tissues and distinct from each other, each peptide binds to specific cytoarchitectonic structures. These studies have been extended in two models: the streptozotocin-induced diabetic rat model of hypoinsulinemia and metabolic derangement, and the Purkinje cell deficient mouse as a model of specific neuronal degeneration. In both models no change in either insulin or IGFs binding was detected.

Chicken embryos are a suitable model for studying the role of insulin, and insulin-like growth factors and their receptors in embryogenesis. We have localized the specific binding of labeled insulin and labeled IGF-I in sections of head and brain as described above. Embryos have been studied from the latter part of organogenesis (day 6 and day 12) through late development (day 18, i.e. 3 days before hatching) and the binding of both ligands was to discrete anatomical regions. In late embryos and adult brain the patterns of labeled insulin and labeled IGF-I binding were quite distinct, in young embryos both ligands showed very similar localization of binding.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 47026-05 DB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Tyrosine-specific protein kinase activity associated with the insulin receptor		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.	Simeon Taylor	Chief, Biochemistry & Molecular Path. DB,NIDDK
Others:	Ronald Margolis	I.P.A., Howard University Wash.,DC
	Catherine McKeon	Senior Staff Fellow DB,NIDDK
	Domenico Accili	Special Volunteer DB,NIDDK
COOPERATING UNITS (if any) Johns Hopkins University, Baltimore, MD (Ann Hubbard) Columbia University, New York (Robert Rees-Jones) University of Catanzaro, Catanzaro, Italy (Nicola Perrotti)		
LAB/BRANCH Diabetes Branch		
SECTION Biochemistry & Molecular Pathogenesis		
INSTITUTE AND LOCATION NIDDK, Bethesda, Maryland		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0
CHECK APPROPRIATE BOXES)		
<input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither		
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>             In the first step in insulin action, insulin binds to its receptor on the surface of the target cell. The <u>insulin receptor</u> is a transmembrane protein which possesses tyrosine-specific protein kinase activity. When insulin binds to the extracellular domain of the receptor, this activates the receptor <u>tyrosine kinase</u> activity. A growing body of evidence suggests that the activation of the receptor's tyrosine kinase is a necessary step in initiating the biological actions of insulin. Accordingly, we have embarked upon a search for intracellular proteins which are substrates for phosphorylation by the receptor-associated tyrosine kinase. We have identified one such substrate in rat liver plasma membranes: a <u>glycoprotein</u> with an apparent molecular weight of 120,000 (pp120). In addition to being a substrate for the insulin receptor, pp120 can be phosphorylated by the receptors for epidermal growth factor and insulin-like growth factor I. pp120 is present in liver from several species, but has not been identified in other tissues. The protein is immunoprecipitated by a monoclonal antibody HA4 which was raised to a glycoprotein present in the bile canaliculus domain of the hepatocyte plasma membrane. This monoclonal antibody has been used to immunoaffinity purify pp120/HA4, and to obtain partial amino acid sequence. Attempts to obtain cDNA clones encoding pp120/HA4 have been initiated.           </p>		

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 47027-04 DB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Use of SMS 201-995 in Hormone Secreting Tumors

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	P. Gorden	Director		NIDDK
Others:	R. F. Arakaki	Senior Staff Fellow	DB,	NIDDK
	A. Shuldiner	Senior Staff Fellow	DB,	NIDDK

## COOPERATING UNITS (if any)

B. Weintraub Chief, MCNEB, NIDDK

## LAB/BRANCH

Diabetes Branch

## SECTION

Clinical and Cellular Biology Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

- |  |  |                                      |
|--|--|--------------------------------------|
| <input checked="" type="checkbox"/> (a) Human subjects | <input type="checkbox"/> (b) Human tissues | <input type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors                   |  |                                      |
| <input type="checkbox"/> (a2) Interviews               |  |                                      |

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

We have studied the use of the long-acting somatostatin analogue, SMS 201-995, in patients with acromegaly, TSH secreting pituitary tumors, glucagonomas and insulinomas. These studies have defined 1) an appropriate dose and schedule for control of TSH secreting pituitary adenoma and its resultant hyperthyroidism, 2) an appropriate subgroup of acromegalic patients in whom this analogue, given thrice daily, controls GH hypersecretion: 3) the effects of the drug in glucagonoma syndrome in terms of control of glucagon hypersecretion and correction of hypoaminoacidemia. Our current studies have focused on the long term use of this agent in acromegaly and patients with TSH secreting tumors and the correlation of hormonal effects with symptomatic benefit. In addition, our studies indicate that all patients develop thickened bile accumulation in the gallbladder while receiving treatment, which may progress to gallstones.

## ANNUAL REPORT OF THE CLINICAL HEMATOLOGY BRANCH

National Institute of Diabetes and Digestive and Kidney Diseases

### Study of Immunology of Blood Cell Deficiencies

**Objectives:** To study the immunochemistry of immune disorders affecting blood cells and effects of the immune reactions on cellular physiology, biochemistry and in vivo cellular kinetics.

**Methods employed:** Techniques of quantitative immunochemistry, including preparation and physiocochemical characterization of purified antibodies and antigens; microanalyses for nitrogen, histamine, and alkaloid drugs; quantitative measurements of complement fixation, cellular agglutination and precipitin reactions; immunoelectrophoresis; methods of provoking antibody responses in man and animals; and isotopic and fluorescent labelling applied to antigens and antibodies. Tissue culture techniques; red cell agglutination techniques; lymphocyte transformation tests; autoradiography; electron microscopy; and high pressure liquid chromatography for analyses of drugs and synthesized analogs. Methods of hemagglutination involving attachment of various antigens to erythrocytes by chemical coupling. Methods of cell membrane component analysis involving cellulose transfer techniques, immunoprecipitation, isoelectric focussing, electroelution, microtiter enzyme immunoassays, and anion exchange chromatography.

Major findings:

#### 1. a New Autoimmune Platelet Disorder

##### Findings:

A patient referred with an 8 month history of obscure chronic severe mucosal bleeding was found to have an autoantibody which binds to the platelet surface causing inhibition of platelet aggregation and secretion. The antibody is transferrable to normal platelets, conferring the same functional defect. The target antigen is the platelet membrane  $Ca^{++}$  dependent glycoprotein (GP) complex IIb/IIIa, not IIb or IIIa alone. Despite high quantities of surface-bound antiplatelet antibody, the circulating platelet count is normal. Lowering antibody titer by extensive plasma exchange followed by high dose adrenocorticosteroids normalized the patient's platelet function.

##### Planned work:

a. Currently experiments are underway to determine whether associated nephrosis is related to immune complex deposition in the kidney or to direct binding of antibody to some element of

kidney shared with platelets, utilizing fresh-frozen normal kidney, purified patient's antibody and immunostaining.

b. The antibody is currently being used to fully characterize the nature of the GP IIb/IIIa epitope because this will be key to further understanding involvement of specific platelet membrane structure in response to physiologic agonists. We have already found that functional abnormalities of Glanzmann's thrombasthenia platelets, which lack GPIIb/IIIa are not as severe as those induced by the patient's antibody.

## 2. A Unique Immunoglobulin-binding Protein from Platelets

### Findings:

We have identified an intracellular platelet protein of 95 kD that binds immunoglobulin present in most normal sera. The 95 kD protein reacts with several classes of normal immunoglobulin (IgG, IgA, and IgM) via the Fab domain and in cytoplasm it appears to exist as a complex with albumin. In these respects it has attributes of the immunoglobulin-binding proteins Streptococcal protein G and Staphylococcal protein A. We have purified the 95 kD protein by anion-exchange chromatography and electroelution from native (non-SDS) gels. Although a highly pure protein has been prepared, amino acid sequencing by automated Edman degradation was not possible because the N-terminal amino acid was blocked, but work is progressing on the protein for possible sequencing of a major proteolytic fragment.

### Planned work:

a. Since platelets have no protein synthesizing capacity, the 95 kD protein could be produced in the stem cell megakaryocyte or could be acquired from outside the cell. Experiments designed to test the possibilities are contemplated.

b. The immunoglobulins that combine with the 95 kD protein are as interesting as the protein itself with respect to Fab specificity responsible for interactions. We plan to purify such immunoglobulins by elution from solid-phase 95 kD protein and study specific binding characteristics.

c. Since the function of the 95 kD protein is not known, we plan to further our observation on diseased states associated with elevated platelet-associated Ig's to determine whether there is correlation with 95 kD-reactive Ig's. This may provide clues concerning the possible role of the 95 kD protein in transfer of Ig's from plasma to platelet alpha granules.

d. Purification of the 95 kD protein with special buffers and antioxidants will be performed to avoid modification of the native protein and possibly prevent a blocked terminal amino acid that has interfered with sequencing. Of special interest would be

comparison of the 95 kD protein sequence with that of Staph protein A and Strep. protein G which have binding characteristics similar to those of the 95 kD protein.

### 3. Experimental Therapy of Immune Neonatal Thrombocytopenia

#### Findings:

We participated in a nation-wide study of prophylactic therapy for immune neonatal thrombocytopenia which was completed this year. Of 25 cases referred to us by local physicians, 7 were found to be caused by alloimmune sensitization to PlA<sup>1</sup> antigen by serologic studies in our laboratory, 1 to possible HLA sensitization, and the others to non immunologic cause, such as eclampsia, fetal asphyxia, or sepsis. The national combined results on over 150 cases indicate that adrenocorticosteroids that cross the placenta and I.V. IgG given to the mother for a 2 week antepartum period prevent fetal hemorrhage at birth when alloimmunity is the cause of neonatal thrombocytopenia. A final report on the national study is forthcoming.

### 4. Pathophysiology of Post-transfusion Purpura (PTP)

#### Findings:

We first described PTP which is a disease caused by alloantibodies against transfused platelet antigens. The sensitized patient develops thrombocytopenia 5 to 8 days post-transfusion. We proposed that adsorption of antigen-antibody complexes on the patient's platelets, involving soluble antigen leached from transfused platelets, was the basis for thrombocytopenia. By quantitative measurements of platelet alloantigens in plasma we found that approximately 1% of cellular antigens are in solution and exchangeable with antigen on the platelet plasma membrane. Antigen-antibody complexes formed with antibodies from cases of PTP are adsorbed onto platelet surfaces in amounts equal to adsorption of antigen alone. These amounts can account for the occurrence of PTP.

#### Planned Proposed Work:

We will apply a sensitive radioimmunoassay we developed for measuring free and adsorbed alloantigen-antibody complexes in attempts to detect antigen and/or complexes in plasma of patients with PTP. Detection of their immune factors would establish the mechanism of PTP and potentially lead to improved therapy.

## Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis

Objective: To study reactions and interactions of coagulation factors in vitro and in vivo, to define further the nature of the blood coagulation mechanism, and to study the physiology and biochemistry of platelet responses to agonists causing exocytosis and aggregation. These studies are aimed at determining factors of significance in the pathogenesis of diseases of hemorrhage and thrombosis and developing better forms of therapy for these diseases.

Methods Employed: Methods of protein purification and characterization, techniques of enzymology, proteolytic enzymes and their inhibitors, kinetic analyses of enzyme reactions, procedures for quantitative measurement of various clotting factors, pharmacologic and physiologic techniques applied in man and animals, and assessment of metabolic pathways of blood cells with radioactive substrates. Tissue culture techniques, pharmacologic and physiologic studies of cellular secretion, and platelet aggregation. SDS-PAGE molecular weights, amino acid composition of purified proteins, immunologic RIA, fluorescent, Western Blot, ELISA and other quantitative and qualitative methods of identifying specific epitopes with antibodies, HPLC, affinity columns, immunoprecipitation, and isoelectric focusing.

### Major Findings:

#### 1. A Novel Mechanisms for Activating Platelets and Other Cells Independent of G-Proteins

Last year we reported evidence that diisothiocyano-2,2' disulfonic acid stilbene (DIDS) was a potent activator of platelets; and that other amine-specific crosslinking agents with a 12-15Å span also activated platelets, albeit more weakly. This year we identified the apparent receptor(s) for DIDS by 1) crosslinking <sup>3</sup>H-methylamine to platelets with DIDS, 2) binding and eluting solubilized platelet membrane proteins to monovalent DIDS (SITS) affinity columns, and by 3) linking 3H-DIDS to platelets. Monovalent DIDS eluate contained a ~66,500 and ~42,000 kD protein on SDS-PAGE; and the other methods which permitted crosslinkage revealed additional proteins of 83,000, 100,000, and 200,000 kD, suggesting multimer formation. These 2 receptor proteins differ from known major platelet membrane glycoproteins.

Platelet aggregation and secretion induced by DIDS and thrombin are similar in that the responses are not effected by apyrase, indomethacin or low extracellular Ca++ and proceed in saponin-permeabilized cells. In <sup>32</sup>P labeled platelets, DIDS, like thrombin, induces phosphorylation of proteins with MW's of 18, 20, 37 and 43. However, in contrast to inhibition of thrombin-induced serotonin secretion in permeabilized cells by 0.1 mM GDP-beta-S,

DIDS-induced secretion is not affected. Therefore DIDS activates platelets independently of eicosanoid production, ionic fluxes and G-protein/receptor interaction. However, DIDS activation is inhibited by PGE<sub>1</sub> and by staurosporine, a protein kinase inhibitor. This data suggests that DIDS acts directly through a protein kinase to activate platelets. In this respect it is similar to epidermal growth factor (EGF) and insulin, two agonists whose receptors upon dimerization by the agonist become active kinases. DIDS activation of platelets may be a model for a yet undescribed platelet hormone or growth factor agonist.

#### Planned Work:

a. To further characterize DIDS receptor(s) by a variety of protein purification and identification procedures currently in use in our laboratory. (See Methods Employed.)

b. To determine activation responses to DIDS of other cell types, particularly granulocytes and lymphocytes and compare characteristics of membrane receptors and signal-transfer biochemistry with those of platelets.

c. To determine characteristics of DIDS-activated lymphocytes for potential therapeutic applications.

#### 2. Modulation of Platelet Function by Peptides Derived from Mucosal Secretory Proteins.

In collaboration with Dr. A. Mukherjee we have studied the effects on platelets of bioactive secretory peptides from uteroglobin (UTG), secreted by progestational uterus, and from lipocortin, a corticosteroid-induced protein. Both peptides inhibit phospholipase A<sub>2</sub> (PLA<sub>2</sub>). Because platelets depend in part on products of PLA<sub>2</sub> activity for activation we studied the effects of these peptides and their nonapeptide and tetrapeptide derivatives on platelets.

The active site for inhibition of PLA<sub>2</sub> has been localized to a nonapeptide, MQMKVLD (P<sub>1</sub>), which is partially homologous to a nonapeptide, HDMNKVLD (P<sub>2</sub>), in lipocortin which also inhibits PLA<sub>2</sub>. P<sub>1</sub> and P<sub>2</sub> share an identical tetrapeptide, KVLD (P<sub>4</sub>). The function of P<sub>4</sub> is not known but its presence in P<sub>1</sub> and P<sub>2</sub> is required for phospholipase A<sub>2</sub> inhibition, although P<sub>4</sub> alone does not inhibit this enzyme

Platelet activation by thrombin or ADP was inhibited by all peptides. The mechanism of platelet aggregation inhibition was found to differ for nonapeptides and P<sub>4</sub> and to be dependent on whether platelets are thrombin- or ADP-activated. All three peptides decrease thrombin esterolytic activity and thereby inhibit thrombin-induced platelet activation. P<sub>1</sub> decreases ADP-induced aggregation and serotonin secretion by inhibiting PLA<sub>2</sub>

whereas P4 decreases only aggregation by blocking fibrinogen binding to activated platelets. P4 therefore has the same activity as the well known RGDS recognition sequence common to many attachment proteins. The P4 sequence in P1 may affect the interaction of P1 with platelets since the presence of P4 potentiates P1 inhibition of platelet activation.

Planned Work:

a. Since P4 (KVLD) resembles RDGS by having a positive and negative charge in close proximity, synthesis of peptides with similar characteristics but different sequences may provide more potent "anti platelet" compounds of potential usefulness in thrombotic disorders.

b. By combining P1 or P2 type structures that have potent anti-PLA2 activity with tetrapeptides that have anti-fibrinogen binding activity, potential therapeutically useful anticoagulants with dual inhibitory properties are possible. Peptide synthesis is currently under way.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 51,000-31 CHB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Study of Immunology of Blood Cell Deficiencies

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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CHB:NIDDK

Others: Diane M. Reid

Senior Staff Fellow

CHB:NIDDK

J. Vostal

Research Fellow

CHB:NIDDK

Charles E. Jones

Chemist

CHB:NIDDK

COOPERATING UNITS (if any)

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LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2

PROFESSIONAL:

1.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects

☐ (b) Human tissues

☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Decreased platelets (thrombocytopenia) is a common cause of clinical hemorrhage. Immunologic abnormalities are the basis for most thrombocytopenias. We have studied the following disorders to further understand mechanisms of immune cellular injury and to develop new diagnostic tests and forms of therapy: autoimmune idiopathic thrombocytopenic purpura; alloimmune and autoimmune neonatal purpura; drug-induced immune purpura; thrombocytopenia of infection; and post-transfusion purpura. The following was accomplished the past year. We have further purified and characterized a platelet 95 kD immunoglobulin-binding protein that we discovered. This protein is the first eukaryotic cell constituent identified with properties similar to Staph. protein A or Strep. protein G. We are currently analyzing the amino acid sequence of the 95 kD protein with the expectation of preparing cDNA. We defined a new autoimmune disease in a patient referred with obscure severe hemorrhage. The unique antibody is directed at a platelet surface glycoprotein complex, is transferrable to normal platelets and affects both platelet secretion and aggregation. The unique epitope recognized by the antibody provides new insight into mechanism of agonist-receptor interaction in platelets. On the basis of testing 18 cases of alloimmune neonatal thrombocytopenia we have established the value of treating mothers antenatally with adrenocorticosteroids and I. V. IgG to prevent fetal hemorrhage at birth. Kinetics of alloantigen exchange between platelets and plasma antigen-antibody complex adsorption by circulating platelets has explained development of post-transfusion purpura, a syndrome we initially described.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 51,001-31 CHB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: N. R. Shulman Chief

Others:	Diane M. Reid	Senior Staff Fellow	CHB:NIDDK
	J. Vostal	Research Fellow	CHB:NIDDK
	Charles E. Jones	Chemist	CHB:NIDDK

## COOPERATING UNITS (if any)

A. B. Mukherjee (NICHD)

## LAB/BRANCH

Clinical Hematology Branch

## SECTION

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2

## PROFESSIONAL:

1.5

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The biochemistry and cellular physiology of platelet secretion has many attributes in common with other secretory cells. Platelet membrane glycoproteins are known to be major factors involved in cell-cell recognition, adhesion, and secretion; but peptide sequences of significance in the various cellular reactions, the biochemistry of signal transmission and the intracellular chain of events leading to activation are only partially clarified.

We have discovered a class of compounds that initiate cellular secretion by cross-linking amine groups with a span of 12-15Å. Linkage occurs with cell membrane proteins of MW ~42,000 and ~66,500 kD plus apparent multimers of 100,000, 140,000 and 200,000 kD. Linkage causes protein kinase activity which phosphorylates proteins with MW's of 18, 20, 37 and 43 kD. Using a variety of activators and inhibitors to evaluate specific metabolic pathways, we have found that the cross-linked receptor develops protein kinase activity similar to that developed by epidermal growth factor and insulin receptors.

The peptide sequence, RGDS, in certain platelet membrane proteins has been identified as an important "recognition peptide" for physiologic agonists. A newly recognized mucosal peptide found in man and other mammals (uteroglobin) has antiinflammatory activity. We found the sequence, KVLD, in uteroglobin to inhibit platelet aggregation by preventing fibrinogen binding to platelets, as does RGDS. However, the sequence MQMKVLDs of uteroglobin which incorporates KVLD, does not inhibit fibrinogen binding, but inhibits phospholipase A2, and as a consequence, platelet function. These observations are a basis for designing synthetic peptides with more potent anti-platelet activity for potential use in therapy of thrombotic diseases as well as in analyzing mechanisms of intermolecular reactions.

ANNUAL REPORT OF THE GENETICS AND BIOCHEMISTRY BRANCH  
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

Biochemical Genetics Section

Dr. Proia and his colleagues have continued their studies of the lysosomal enzyme  $\beta$ -hexosaminidase (a deficiency of which is responsible for Tay-Sachs disease). This enzyme is made up of two subunits  $\alpha$  and  $\beta$ ; last year they determined the structure and function of the five potential glycosylation sites of the  $\beta$  subunit, this year they have extended their studies to the  $\alpha$  subunit. All three potential glycosylation sites were utilized and only one was phosphorylated. The lack of any one of the oligosaccharide chains (removed by site-directed mutagenesis of the appropriate asparagine) did not dramatically affect catalytic activity. In contrast, the removal of all three oligosaccharide chains resulted in the synthesis of an extremely large, insoluble, disulfide-linked polypeptide complex that was trapped in the endoplasmic reticulum.

In a different project Dr. Proia and his colleagues have identified a single base change in the  $\beta$ -hexosaminidase  $\alpha$ -subunit gene as the mutation in Ashkenazi Jewish patients with the adult form of Tay-Sachs disease. The mutation results in the substitution of Ser for Gly-269.

Molecular Genetics Section

Dr. Ackerman and collaborators have continued their work on the mode of action of the Aspergillus toxin Alpha-sarcin and related toxins. They have shown that the toxins  $\alpha$ -sarcin, ricin, Shiga toxin and Shiga-like toxin all inactivate protein synthesis in cells by attacking a highly conserved region near the 3'-end of 28S ribosomal RNA. They have recently show that microinjection into Xenopus oocytes of oligodeoxynucleotides complementary to the same region of ribosomal RNA also abolished protein synthesis.

On a second project Dr. Ackerman and his colleagues have extended their results showing that oocytes efficiently repair microinjected DNA containing pyrimidine dimers by using a variety of new assays to document the repair.

Dr. Hsieh and her colleagues have established that recombinases from E. coli, Drosophila and human cells can form stable joint molecules between a linear duplex and a single strand when the region of homology shared by these two substrates can be very short, as short as 13 base pairs in the case of the eukaryotic proteins. They then showed that the recognition of sequence homology does not require melting of the duplex DNA. In addition, thermal stability studies of the joint molecules formed with short regions of homology

demonstrated that these joints do not have an unpaired strand that can participate in branch migration. On the basis of these observations they have proposed a novel structure for the protein-free intermediate in recombination: a three- stranded DNA.

Dr. Camerini-Otero and his colleagues have demonstrated that RecA, the E. coli recombinase, can recognize and pair regions of homology as short as 6 base pairs. This recognition can be detected in the form of ternary complexes between the recombinase, a duplex DNA and an oligonucleotide. These complexes are very stable and can be footprinted with restriction endonucleases.

Dr. Camerini-Otero's group has further extended their studies of the possible involvement of three-stranded DNA in recombination by investigating DNA in the form of a triple-helix (a triplex). They have shown that a variety of sequence motifs can be used in non-enzymatically forming triplexes. In particular, they have extended the range of possible triplexes by forming them intermolecularly, under physiological conditions and, most importantly, with one pyrimidine and two purine strands. They have also shown that these triplexes are very stable and have melting temperatures comparable to duplexes of the same length and sequence.

## DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 52008-10 GBB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Gene Expression and Human Genetics

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	R. D. Camerini-Otero	Chief	GBB, NIDDK
Others:	M. Hsieh	Senior Staff Fellow	GBB, NIDDK
	A. Eisen	Med. Staff Fellow	GBB, NIDDK
	C. S. Camerini-Otero	Med. Staff Fellow	GBB, NIDDK
	F. Mills	Guest Researcher	GBB, NIDDK
	R. Kiyama	Visiting Fellow	GBB, NIDDK
	R. Gardner	Med. Staff Fellow	GBB, NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Genetics and Biochemistry Branch

## SECTION

Molecular Genetics Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

6.0

## PROFESSIONAL:

5.5

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In order to dissect the biochemical steps involved in genetic recombination we have chosen to focus on a key early step: strand exchange between homologous parental DNAs. The product of this strand exchange reaction is a joint molecule composed of a single-strand circle joined to one end of a linear duplex. Three proteins responsible for this step have been purified: *uvx* from phage T4; *RecA* from *E. coli*; and *rec1* from *U. maydis*.

Over the last two years we have reported the partial purification and characterization of similar strand-exchange proteins or recombinases from nuclear extracts of human cells and tissues, and embryos of *D. melanogaster*.

Recently, we have shown that the structure of the protein-free intermediate in stand-exchange is most likely that of a three-stranded nucleic acid. Over the last year we have concentrated on forming triplex structures from certain nucleic acid sequences. Using defined sequences we have been able to extend the range of sequences that can non-enzymatically form triplexes. Although, for the most part, such triplexes have been described between two polypyrimidine (Py) strands and one polypurine (Pu) strand, we have been able to show that stable structures can be formed under physiological conditions between two Pu stands and one Py strand.

In order to study the mechanism of strand exchange we have devised a novel assay to detect homology-dependent ternary interactions between single strand DNA, double strand DNA and the *E. coli* *RecA* recombinase protein. Surprisingly, stable ternary complexes can be efficiently formed with regions of homology shorter than a helical turn of DNA.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 52011-05 GBB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Toxins and DNA Repair in <i>Xenopus</i> Oocytes		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	Eric Ackerman	Senior Staff Fellow      GBB, NIDDK
Others:	Shailendra K. Saxena	Visiting Fellow      GBB, NIDDK
	Jitendra K. Saxena	Visiting Associate      GBB, NIDDK
COOPERATING UNITS (if any)		
LAB/BRANCH Genetics and Biochemistry Branch		
SECTION Molecular Genetics Section		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.25	3.0	0.25
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>I. We showed that the toxins <math>\alpha</math>-sarcin, ricin, Shiga toxin and Shiga-like toxin all inactivate protein synthesis in cells by attacking a highly conserved region near the 3'-end of 28S ribosomal RNA. All of these toxins were microinjected into <i>Xenopus</i> oocytes. We have recently shown that microinjection of deoxyoligonucleotides complementary to the same region of 28S RNA attacked by these toxins abolishes protein synthesis.</p> <p>II. During early development <i>Xenopus</i> replicates its DNA nearly as fast as <i>E. coli</i> in log phase; perhaps indicating that oocytes may be an excellent source of DNA repair activity. We have investigated pyrimidine dimer repair by microinjecting UV-irradiated DNA into oocytes and assaying for repair using 2 methods: (1) Transformation of repair deficient <i>E. coli</i> mutants with the microinjected DNA; (2) Absence of pyrimidine dimers using UV-endonuclease and denaturing agarose gels.</p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 52012-05 GBB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structure-Function Relationships of Lysosomal Enzymes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Richard L. Proia

Senior Staff Fellow GBB, NIDDK

Others: Ruth Navon

Visiting Associate GBB, NIDDK

Gabrielle Weitz

Visiting Fellow GBB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Genetics and Biochemistry Branch

SECTION

Biochemical Genetics Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2.75

PROFESSIONAL:

2.5

OTHER:

0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects

☒ (b) Human tissues

☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

I. We have characterized the glycosylation sites of human  $\beta$ -hexosaminidase B and have determined the effect of individual oligosaccharides on folding, catalytic activity, phosphorylation and transport. All three sites were normally glycosylated and the third oligosaccharide was predominantly phosphorylated. The lack of any one of the oligosaccharide chains did not dramatically affect catalytic activity and, thus, the folding of the polypeptide. In contrast, when all three oligosaccharides were absent the polypeptide aggregated into an extremely large, insoluble, disulfide-linked complex which was not transported out of the endoplasmic reticulum.

II. We have identified a single base change in the  $\beta$ -hexosaminidase  $\alpha$ -subunit gene in Ashkenazi patients with the adult form of Tay-Sachs disease that results in the substitution of Ser for Gly-269. We have also found the same Gly-269 to Ser mutation in 6 patients from 4 different non-Jewish families.

III. We have identified a three base pair deletion in exon 8 in the  $\alpha$ -subunit gene in a Moroccan Jewish patient with infantile Tay-Sachs disease. This mutation was in compound heterozygosity with a second unidentified mutation. The three base pair deletion was found in five out of eight Moroccan Jews carrying a Tay-Sachs disease allele.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 52014-02 GBB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) CD4 Receptor Structure/Function Project		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)  <div style="display: flex; justify-content: space-between;"> <div>           PI: R. Daniel Camerini-Otero            Others: Richard L. Proia         </div> <div>           Chief,            Senior Staff Fellow,         </div> <div>           GBB, NIDDK            GBB, NIDDK         </div> </div>		
COOPERATING UNITS (if any)  <div style="display: flex; justify-content: space-between;"> <div>Cynthia Tift</div> <div>Medical Staff Fellow</div> <div>OD, CC</div> </div>		
LAB/BRANCH Genetics and Biochemistry Branch		
SECTION Molecular Genetics Section		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 1.3	PROFESSIONAL: 1.2	OTHER: 0.1
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div> <input type="checkbox"/> (a) Human subjects  <input type="checkbox"/> (a1) Minors  <input type="checkbox"/> (a2) Interviews         </div> <div> <input type="checkbox"/> (b) Human tissues         </div> <div> <input checked="" type="checkbox"/> (c) Neither         </div> </div>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>CD4, a T cell surface glycoprotein and member of the immunoglobulin supergene family, is not only a receptor for antigen recognition and immune system activation, but also the receptor for the human immunodeficiency virus (HIV). Studies have shown that the presence of CD4 is not only necessary but sufficient to render cells susceptible to HIV infection. Several reports have demonstrated that soluble CD4 is able to selectively inhibit and neutralize HIV binding and infection of CD4+ cells. Recent reports have localized the site of HIV binding to the V1-like region near the amino-terminus of the protein.</p> <p>Glycosylation of some glycoproteins has been shown to be required for their processing and transport to the cell surface. The primary sequence of CD4 indicates two potential glycosylation sites. A recent report utilizing tunicamycin to block glycosylation of T4+ cells suggests that glycosylation is necessary for cell surface expression of the receptor.</p> <p>By using site-directed mutagenesis we have previously created a series of glycosylation mutants of CD4 and by transient expression in COS 1 cells have shown that both glycosylation sites are utilized in the expression of CD4. We have more recently isolated stable mouse cell lines transfected with CD4 glycosylation mutant DNA.</p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 52015-01GBB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Molecular Studies of Protein-DNA Interactions		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: Others:	P. Hsieh R. D. Camerini-Otero C. S. Camerini-Otero	Sr. Staff Fellow Branch Chief Medical Staff Fellow   GBB, NIDDK GBB, NIDDK GBB, NIDDK
COOPERATING UNITS (if any)		
LAB/BRANCH Genetics and Biochemistry Branch		
SECTION Molecular Genetics Section		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.55	1.4	0.15
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>We have studied early steps in homologous recombination carried out by a human recombinase activity isolated from nuclear extracts of HeLa cells. These early steps involve the recognition by recombinases of DNA sequence homology residing on two DNA molecules and the subsequent pairing of these sequences resulting in joint molecule formation.</p> <p>As few as 13 bp of sequence homology is recognized by the human recombinase protein in a joint molecule assay. The recombinase can pair a linear duplex with a homologous single-strand DNA which is either linear or circular. The reaction leads to the formation of stable joint molecules in which the two DNA substrates are joined by a region of hydrogen bonding.</p> <p>We have demonstrated that sequence recognition by recombinases does not require melting of the duplex DNA. Thermal stability assays demonstrate that the joint molecules do not have an unpaired strand that can participate in branch migration. On the basis of these observations, we conclude that a triple-stranded DNA structure is an intermediate in recombination. Efforts are underway to further characterize this novel protein-DNA complex.</p>		

Annual Report of The Digestive Diseases Branch  
National Institute of Diabetes, and Digestive and Kidney Diseases

SUMMARY OF BRANCH ACTIVITIES

The Digestive Diseases Branch has three sections (Section on Gastroenterology, Section on Cell Biology and Liver Diseases Section). Detailed summaries of the activities of each section follow. All three sections are engaged in investigations of basic biologic processes (e.g., hormone action, membrane transport, cellular and humeral immunology) and are attempting to apply this information to understand the pathophysiology of various disorders involving the liver, pancreas and gastrointestinal tract. All three sections are also involved in attempts to improve therapy of clinical disorders such as neoplasms associated with overproduction of gastrointestinal hormones, hepatitis and fulminant hepatic failure.

SECTION ON GASTROENTEROLOGY

The Digestive Diseases Branch has cared for approximately 150 patients with Zollinger-Ellison syndrome (ZES, gastrin-producing neoplasm, hypergastrinemia and increased secretion of gastric acid). All current patients are being treated with oral medication that inhibits gastric acid secretion.

During this past year we completed a study of the efficacy of chemotherapy with 5-FU, adriamycin and streptozotocin in ten patients with metastatic gastrinoma. Our results show that four patients showed a favorable response, that this response was short-lived and that these four patients did not live longer than the six patients who did not respond.

Recently Sandoz Pharmaceutical Company developed a long-acting analogue of somatostatin (SMS 201-995, Sandostatin) that appears to have potentially beneficial effects in patients with functioning islet-cell tumors. There is a spectrum of response, however, depending on the type of islet cell tumor. In some instances therapy with the somatostatin analogue is clinically beneficial (e.g., reducing the diarrhea in patients with VIPoma), whereas in other situations the somatostatin analogue is not particularly helpful (e.g., reducing gastric acid secretion in patients with gastrinoma).

In the mid 1970's it became clear that oral histamine H<sub>2</sub>-receptor antagonists constituted effective therapy for the hypersecretion of gastric acid that occurs in patients with Zollinger-Ellison syndrome. In the early 1980's forms of histamine H<sub>2</sub>-receptor drugs became available that were suitable for intravenous administration. Intravenous histamine H<sub>2</sub>-receptor antagonists are particularly useful for treating patients with Zollinger-Ellison syndrome under conditions when therapy with oral drug is not practical (e.g., abdominal surgery, or nausea and vomiting accompanying antineoplastic chemotherapy).

In the early 1980's Hassle Pharmaceutical Company developed a drug (omeprazole) that inhibits gastric acid secretion by inactivating the proton pump in the gastric parietal cell. We were among the first groups to show that omeprazole constitutes particularly effective therapy for the hypersecretion of gastric acid that occurs in patients with Zollinger-Ellison syndrome. During this past year we have completed a 4-year follow-up study of patients with Zollinger-Ellison syndrome being treated with omeprazole and report that omeprazole constitutes effective, safe, long-term therapy for patients with Zollinger-Ellison syndrome.

Most patients with Zollinger-Ellison syndrome have a gastrin-secreting neoplasm (gastrinoma) in or near the pancreas. Sometimes, however, patients with Zollinger-Ellison syndrome may have a gastrinoma in a site far removed from the region of the pancreas. During this past year we cared for a patient with Zollinger-Ellison syndrome who had a gastrinoma in her organs. Resection of the neoplasm cured the patient of her Zollinger-Ellison syndrome.

Recently a number of antagonists have been developed that interact with cell surface receptors for gastrointestinal peptides. In most instances these receptor antagonists are highly specific and interact with only one class of receptors. During the past year, however, we examined an analogue of substance P that functions as a competitive, reversible receptor antagonist at receptors for substance P and related peptides, at receptors for bombesin and related peptides, and at receptors for cholecystokinin and related peptides.

Previous studies have reported that gastric smooth muscle cells possess receptors that interact with cholecystokinin. Recently we have shown that these receptors on gastric smooth muscle cells are actually gastrin receptors and like gastrin receptors on other target tissues, have a high affinity for gastrin and cholecystokinin and interact with gastrin-specific receptor antagonists.

Jean Martinez of Montpellier, France synthesized an analogue of the C-terminal heptapeptide of cholecystokinin (CCK) that we have found to be particularly useful for examining receptors for CCK on pancreatic acinar cells. Pancreatic acinar cells possess two classes of receptors that interact with CCK - one class has a high affinity for CCK and mediates maximal stimulation of enzyme secretion; the other class has a low affinity for CCK and mediates submaximal stimulation of enzyme secretion. The Martinez analogue (CCK-JMV-180) possesses the interesting properties of being a full agonist at high affinity CCK receptors and a competitive antagonist at low affinity CCK receptors.

Until recently it was believed that mobilization of cellular calcium mediated the action of CCK on high affinity CCK receptors on pancreatic acinar cells. Using CCK-JMV-180 we have shown that mobilization of cellular calcium is associated with occupation of low affinity CCK receptors on pancreatic acinar cells. The mediator of the action of CCK at high affinity CCK receptors on pancreatic acinar cells remains to be determined.

First incubating pancreatic acinar cells with CCK reduces the subsequent stimulation of enzyme secretion measured during a second incubation. This desensitization of enzyme secretion is specific for CCK receptors in that it can be blocked by CCK-specific receptor antagonists. Using CCK-JMV-180 we found that CCK-induced desensitization of enzyme secretion is mediated by occupation of low affinity CCK receptors by CCK.

Some investigators have proposed that cyclic GMP can modulate the actions of protein kinase C in pancreatic acinar cells. Recently we showed that although cyclic GMP is increased by a number of different agents acting on pancreatic acinar cells, cyclic GMP does not alter the actions of protein kinase C in pancreatic acinar cells.

During the past year it has become clear that occupation of CCK receptors by CCK can modify the interaction of other peptides with this receptor on pancreatic acinar cells. For example, occupation of low affinity CCK receptors for CCK reduced the affinity, but not the number of bombesin receptors for bombesin. CCK also causes desensitization of bombesin-stimulated enzyme secretion; however, this desensitization of enzyme secretion is independent from the CCK-induced change in bombesin receptor affinity.

Like CCK, carbachol, a muscarinic cholinergic agonist that is resistant to degradation by abolinesterase, also modifies the properties of receptors for other secretagogues on pancreatic acinar cells. Pancreatic acinar cells possess two classes of receptors that interact with VIP - one class has a high affinity for VIP and mediates stimulation of enzyme secretion; the other has a low affinity for VIP. Carbachol causes a 30- to 50-percent decrease in the number of high affinity VIP receptors with no change in the number of low affinity VIP receptors or in the affinities for VIP of either class of receptors. Because pancreatic acinar cells possess so many "spare" high affinity VIP receptors, the carbachol-induced decrease in the number of high affinity VIP receptors is not sufficient to reduce VIP-stimulated enzyme secretion.

Carbachol also modifies receptors for secretagogues other than VIP on pancreatic acinar cells. Pancreatic acinar cells possess two classes of cholinergic receptors that interact with carbachol - one class has a high affinity for carbachol; the other has a low affinity for carbachol. Occupation of the high affinity cholinergic receptors by carbachol reduces the number of high affinity cholinergic receptors but does not alter the number of low affinity receptors. Occupation of low affinity cholinergic receptors by carbachol does not alter the number or affinity of either high affinity or low affinity cholinergic receptors, reduces the number of bombesin receptors, reduces the number of high affinity CCK receptors and does not alter the low affinity CCK receptors. These carbachol-induced changes in receptor number are accompanied by a reduction in stimulation of enzyme secretion by the appropriate secretagogue.

## SECTION ON CELL BIOLOGY

### I. Studies relating to the identification and characterization of receptors for gastrointestinal peptides.

These studies involve the characterization of receptors for gastrointestinal peptides by developing potent, specific antagonists; the development of selective ligands for various receptors for gastrointestinal peptides; the use of the radiolabeled ligands to identify new receptors for gastrointestinal peptides on various tissues, and the characterization of these receptors using ligand binding techniques, cross-linking studies and eventually solubilization and structural determination. In this category we have performed the following studies.

#### A. Development and characterization of bombesin receptor antagonists.

Significant advances have been made in collaboration with Dr. D.H. Coy (Tulane University) in discovering new, potent bombesin receptor antagonists. Five chemically different classes of bombesin receptor antagonists have now been identified in our studies. A number of these analogues have approximately equal affinity to bombesin for the bombesin receptor, have been shown to function as competitive antagonists of the ability of bombesin to stimulate growth of 3T3 cells and to stimulate enzyme secretion from pancreatic acinar cells and preliminary studies suggest that they are also extremely potent inhibitors of the ability of bombesin to cause pancreatic enzyme secretion in in vivo studies. Therefore a number of these antagonists may prove useful in defining the physiological role of bombesin in various processes. In addition, bombesin has been shown to be a potent growth factor for human small cell lung cancer cells and therefore some of these antagonists may be useful for inhibiting the growth of this tumor.

In addition, studies of previously described bombesin receptor antagonists have been extended in collaborative studies (E. Sausville, NCI; G. Della Fave, Univ. La Sapienza, Rome; T.W. Moody, George Washington University, Wash., D.C.) and a number of these analogues have been shown to antagonize the actions of bombesin in vivo and in vitro on human small cell lung cancer cells, in the CNS of the rat and in guinea pig gastric smooth muscle cells.

#### B. Development and/or characterization of receptor antagonists for other gastrointestinal peptides.

In collaboration with Dr. D.H. Coy (Tulane University) a novel class of substance P receptor antagonists has been identified which has enhanced specificity for substance P receptors. One analogue, Leu<sup>10</sup>  $\psi$ (CH<sub>2</sub>NH)Leu<sup>11</sup>-substance P had greater than 50 times high affinity for substance P than for bombesin receptors. In comparison [D-Arg<sup>1</sup>,D-Trp<sup>7,9</sup>,Leu<sup>11</sup>]-substance P, which is a currently, widely used substance P receptor antagonist, has only a 1.5-fold higher affinity for substance P receptors than bombesin receptors.

A new class of CCK receptor antagonists, the 3-(benzoyl-amino)benzodiazepine analogues such as L-365,260, has been recently developed by the Merck Co. L-365,260 was shown in our studies to have a 70-fold higher affinity for gastrin type CCK receptors on gastric smooth muscle cells or guinea pig pancreatic acini than for pancreatic type CCK receptors on guinea pig pancreatic acini. In contrast, in our studies the 3-(acylamino)benzodiazepine analogue, L-364,718, had a 125-fold higher affinity for pancreatic than gastrin type CCK receptors. These two different classes of CCK receptor antagonists thus distinguish different subtypes of CCK receptors and should be useful for in vivo studies and for studies attempting to differentiate the role of these receptors in various physiological processes.

C. Identification and characterization of receptors for gastrointestinal hormones using various radioligands.

A number of different mammalian peptides which are structurally related to the bombesin such as GRP, neuromedin C and neuromedin B, have been described. However, up to the present only a single class of receptors have been described which interacts with these peptides. In our studies for the first time subtypes of bombesin receptors were identified. Using  $^{125}\text{I}$ -BH-neuromedin B a receptor was identified on rat esophageal muscle that had a high affinity for neuromedin B and bombesin, whereas in rat pancreas using  $^{125}\text{I}$ -[Tyr<sup>4</sup>]bombesin, receptors with high affinity for bombesin and low affinity for neuromedin B were identified. The pancreatic bombesin receptor mediated enzyme secretion by bombesin-related peptides and the esophageal muscle neuromedin B receptor mediated esophageal muscle contraction.

Whereas a considerable number of functional studies provide evidence for CCK receptors on gallbladder muscle and pancreatic acinar cells, because of the small size of the gallbladder in various laboratory animals such as guinea pigs, there are no studies that have directly compared the interaction with these receptors using ligand binding techniques. A tissue section method was developed which allowed comparison of receptors for gastrointestinal hormones on relatively small pieces of tissue. This allowed for the first time a comparison under identical conditions, the receptors mediating CCK-induced gallbladder contraction or pancreatic secretion, two of the main physiological functions of CCK. The ability of a number of CCK receptor agonists as well as a representative member of each class of CCK receptor antagonist was examined for its ability to interact with CCK<sub>A</sub> receptors on gallbladder muscle or pancreatic acinar cells. This study demonstrated these receptors can not be differentiated by existing agonists or antagonists. Furthermore, these studies provided no evidence that they represent different subtypes of CCK<sub>A</sub> receptors as has been proposed by others.

In collaboration with Dr. T.W. Moody (George Washington University) human lung cancer cell lines were found to possess specific receptors for vasoactive intestinal peptide (VIP). These receptors had a high affinity for VIP and low affinity for secretin, and cross-linking studies demonstrated they resembled those described previously on guinea pig pancreatic acinar cells.

Both CCK receptors and gastrin receptors have been described. CCK and gastrin share a common COOH terminal pentapeptide, and therefore interact with each other's receptors. However, CCK and gastrin can exist in both sulfated and nonsulfated forms and when sulfated the sulfation occurs in different positions in the peptides. CCK is sulfated on the tyrosine 7 amino acids from the COOH terminus, whereas gastrin-II is sulfated on the tyrosine in the 6th position from the COOH terminus. The importance of the position of the sulfation was explored for both CCK and gastrin receptors. For both CCK and gastrin receptors, sulfated gastrin or CCK-8 had a higher affinity than the unsulfated peptides. However, the position of the sulfate was much more a critical factor for increasing potency at the CCK receptor than at the gastrin receptor.

## II. Studies related to mechanism of the ability of gastrointestinal peptides to alter cellular function.

These studies involve investigating the mechanism by which various gastrointestinal peptides alter cell function. The studies include the relationship of receptor occupation to subsequent changes in cellular processes, characterization of second messengers including calcium, cyclic nucleotides and the breakdown of polyphosphoinositides, as well as characterization of the more distal steps in cell activation.

Muscarinic cholinergic agents in vitro and in vivo have been shown to be important stimulants of pepsinogen release from chief cells. To gain additional insight into their mechanism of stimulation of pepsinogen release, an isolated enriched (>90% pure) preparation of chief cells from guinea pig stomach was used to study the interaction of muscarinic cholinergic agents with these cells. Ligand binding studies using [<sup>3</sup>H]N-methylscopolamine demonstrated that these cells possessed high and low affinity muscarinic cholinergic receptors. Pharmacologic studies using various specific antagonists demonstrated the receptors were of the M<sub>2C</sub> subtype in that they had a high affinity for 4-DAMP or scopolamine, low affinity for pirenzepine and an even lower affinity for AF-DX-116. Studies comparing the ability of the various muscarinic cholinergic agonists to occupy receptors and stimulate pepsinogen release demonstrate each was 30- to 60-times more potent at stimulating pepsinogen release than occupy high affinity receptors. Studies using the alkylating agent PCM provided evidence that 50-80% of receptors were spare receptors and that occupation of high affinity receptors best correlated with their ability to stimulate pepsinogen release.

CCK has been shown to be widely present in the central nervous system; however, its function or mechanism of action is unknown. In collaborative studies with C. Bondy and H. Gainer (NINCDS) the mechanism of the ability of CCK to regulate oxytocin and vasopressin release from the neural lobe of the pituitary was investigated. The rat neural lobe was found to contain high affinity CCK receptors, occupation of which by CCK or related peptides

stimulate vasopressin or oxytocin release. This action of CCK was independent of extra-cellular calcium, electrical stimulation and blocked by a protein kinase C inhibitor. This study suggests this action of CCK is mediated not by depolarization-induced calcium influx, but by intracellular messengers that activate protein kinase C such as diacylglycerol and calcium.

Recently, the VIP-secretin family of peptides has been found to include a number of different structurally related peptides including VIP, secretin, PHI, PHM, helospectin I and II, helodermin and growth hormone releasing factor. To explore their cellular basis of action their ability to interact with pancreatic acini was explored. All of these peptides were found to interact with both VIP-preferring and secretin-preferring receptors, to activate adenylate cyclase, increase cellular cyclic AMP and to stimulate enzyme secretion. Each of these peptides was a full agonist at both VIP and secretin receptors, but they differed in the potencies for interacting with VIP- or secretin-preferring receptors and increasing cyclic AMP. For the VIP-preferring receptors the relative potencies were VIP > helospectin I = helospectin II = helodermin = rGHRF > PHI = PHM = > hGHRF > secretin and for the secretin-preferring receptors the relative potencies were secretin > helospectin I = helospectin II = helodermin > rGHRF = PHI = PHM > hGHRF. Correlation of the abilities of various peptides to occupy VIP- or secretin-preferring receptors and increase cyclic AMP suggested that all of the actions of these peptides to alter acinar cell function could be explained by their ability to occupy either VIP- or secretin-preferring receptors. This result was confirmed by radiolabeling helodermin, and demonstrating all of <sup>125</sup>I-helodermin binding to pancreatic acinar cells could be explained by it binding to VIP- or secretin-preferring receptors.

## Liver Diseases Section

The Liver Diseases Section is currently responsible for seven principal projects.

### I. Studies Relating to the Pathogenesis of Hepatic Encephalopathy

The abnormal pattern of visual evoked responses (VERs) in animals with hepatic encephalopathy (HE) due to fulminant hepatic failure (FHF) resembles that induced by drugs which promote GABA-ergic neurotransmission, including benzodiazepines (BZs). Furthermore, animals with HE due to FHF exhibit increased resistance to drugs that induce convulsions by decreasing GABA-ergic tone. Ameliorations of HE (both clinical and electrophysiologic) have been induced in animals with FHF by BZ receptor antagonists. Furthermore, Purkinje neurons from rabbits in HE due to FHF exhibited increased sensitivity to depression by agonists of the GABA/BZ receptor complex, including a BZ, and, in contrast to control neurons, exhibited excitation when exposed to BZ receptor antagonists. These findings suggest that in HE due to FHF: (i) There is increased GABA-ergic tone; (ii) Blockading of BZ receptors can ameliorate HE; (iii) BZ receptor antagonists may be of value in the management of HE; and (iv) An endogenous BZ receptor agonist may contribute to HE. Autoradiographic and neurochemical evidence for the existence of such a ligand has been found in the brains of animal models of HE. [E.A. Jones, J. Vergalla, S.H. Gammal, N. Bergasa, B.L. Baker, M. Lisker-Melman, A.S. Basile, P. Skolnick, C. Banner].

### II. Trials of Therapies for Primary Biliary Cirrhosis

Primary biliary cirrhosis (PBC) is a disease of unknown etiology characterized by slowly progressive intrahepatic cholestasis due to non-suppurative, presumably autoimmune, destruction of small bile ducts. As some other autoimmune diseases appear to respond favorably to certain immunosuppressive agents, trials of selected immunosuppressive drugs are being undertaken in patients with PBC. In a randomized controlled trial of chlorambucil therapy, which involved 24 patients, treatment with the drug was associated with a decrease in the rate of increase in serum bilirubin, normalization of elevated serum IgM levels, an improvement in inflammatory cell infiltrate in the liver and a variable degree of bone marrow suppression. These findings strongly suggest that immunosuppressive therapy can retard the progression of PBC. More effective less toxic immunosuppressive regimens are being sought for this disease. Currently the effects of low-dose methotrexate are being evaluated in an open trial. [E.A. Jones, J.H. Hoofnagle, N.V. Bergasa, J. Korenman, T.-L. Fong, A.M. DiBisceglie].

### III. Studies of Cellular Immune Function in Primary Biliary Cirrhosis

The role of abnormal immune mechanisms in the mediation of the hepatobiliary lesion of primary biliary cirrhosis (PBC) is being studied. Recently, with the use of monoclonal antibodies, it has become apparent that CD4 (T4) T cells can be subdivided into subpopulations having unique functions. In particular CD4 positive, Leu-8 positive T cells have been demonstrated to have direct suppressor function, as well as the capacity for inducing CD8 (T8) suppressor cells. In addition, it has been shown that the CD4 positive, Leu-8 positive T cell population is the predominant autoreactive T cell subpopulation

in peripheral blood. CD4+, Leu-8+ T cells from patients with PBC, but not from patients with other liver diseases, have been shown to exhibit a defect in their ability to suppress immunoglobulin synthesis by B cells in vitro. Furthermore the proliferative responses of these cells from patients with PBC to mitogenic stimulation were found to be impaired. However, the defect in proliferative responses did not correlate with the defect in suppression of immunoglobulin synthesis, suggesting that these two defects are due to different mechanisms. The abnormal function of the CD4+, Leu-8+ T cell subpopulation in patients with PBC may play a central role in the defective immunoregulation found in this disease. Exposure of this subpopulation of T cells from patients with PBC to phorbol ester, which activates the protein kinase C pathway, corrects the defective response of these cells to mitogens. Thus abnormal function of the biochemical pathway involving protein kinase C may contribute to the immunological abnormalities exhibited by patients with PBC. [E.A. Jones, R. Moreno-Otero, S.P. James, J.H. Hoofnagle, J. Vergalla].

#### IV. Studies of the Natural History and Treatment of Chronic Type B Hepatitis

A cohort of patients with chronic type B hepatitis is being evaluated and followed to determine the long-term natural history of this common form of chronic liver disease. Selected patients have been entered into therapeutic trials in which antiviral or immunomodulatory agents are being administered. A randomized controlled trial of interferon therapy vs. no treatment is underway. Twenty three patients have been entered and 11 have completed interferon therapy and follow up. Five (45%) responded with loss of HBeAg from serum. In addition, a pilot study is examining the effect of one month of corticosteroid pre-treatment followed by interferon therapy for patients who have previously not responded to interferon therapy alone. [J.H. Hoofnagle, A.M. DiBisceglie, P. Martin, N.V. Bergasa, J. Korenman, M. Lisker-Melman, B.L. Baker, S.H. Gammal, E.A. Jones; not NIH: J. Gerin, M. Sjogren].

#### V. Studies of the Natural History and Treatment of Chronic Non-A, Non-B (Type C) Hepatitis

Patients with well-documented chronic non-A, non-B hepatitis are being evaluated to determine the long-term natural history of this common form of chronic liver disease. A cohort of such patients is available to enable experimental therapies for this disease to be evaluated. A pilot study demonstrated that alpha interferon was effective in normalizing serum ALT activities in a majority of cases. This effect was associated with an improvement in liver histopathology (decreased activity of hepatitis). A prospective randomized, placebo-controlled trial of alpha interferon therapy for chronic non A, non B hepatitis is nearing completion. Forty one patients have completed the initial 6 months blinded phase. Interferon appears to be effective in decreasing serum aminotransferase activities and improving liver histopathology in the majority of cases. [J.H. Hoofnagle, A.M. DiBisceglie, M. Lisker-Melman, N.V. Bergasa, J. Korenman, H.J. Alter, J. Everhart, E.A. Jones; not NIH: Z. Goodman, G. Kuo].

#### VI. Immunologic Studies of Chronic Viral Hepatitis

Immunological factors seem to be important in determining the course and outcome of both acute and chronic viral hepatitis. Furthermore promising

therapies for chronic viral hepatitis have profound effects on immune function and sustained responses to therapy may depend largely on restoration of normal immune responsiveness. The role of immunologic mechanisms in determining the course and ultimate outcome of viral hepatitis is being studied and the effects of antiviral and immunomodulatory therapies on the immune system are being evaluated in patients with chronic viral hepatitis. Serial studies of cellular immune function have been performed on patients with chronic type B hepatitis treated with interferon. [J.H. Hoofnagle, E.A. Jones, A.M. Di Bisceglie, M. Lisker-Melman, R. Moreno-Otero, J. Korenman, J. Ambrus; not NIH: T. Cupps].

#### VII. Studies of the Natural History and Treatment of Duck Hepatitis B Virus Infection

Duck hepatitis B virus (DHBV) infection is a potentially useful experimental model of human hepatitis B virus infection. The ability of new antiviral and immunomodulatory agents to suppress DHBV replication in ducks is being assessed. It is anticipated that the ability of a drug to suppress DHBV replication will be shown to be a satisfactory screening test for new effective therapies for chronic type B hepatitis in man. Care of DHBV-infected ducks as well as methods for obtaining serum and liver tissue from ducks have been standardized. Reproducible assays for quantitating DHBV DNA and DNA polymerase in serum have been established. 2',3'-dideoxynucleosides (DDC, DDA, DDG, DDI) have been shown to be potent inhibitors of DHBV replication and DDI does not appear to cause severe side effects. The effects of DDI will shortly be investigated in humans with hepatitis B infection. [E.A. Jones, P. Martin, H. Robcis, R. Miller, H. Mitsuya, S. Broder, J.H. Hoofnagle].

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		<b>PROJECT NUMBER</b>  Z01 DK 53001-19 DDB
<b>PERIOD COVERED</b> October 1, 1988 to Sept. 30, 1989		
<b>TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)</b> Studies of Membrane Function		
<b>PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)</b>		
PI:	Jerry D. Gardner	Chief DDB, NIDDK
Others:	R.T. Jensen	Chief, Cell Biology Sect. DDB, NIDDK
	P.N. Maton	Visiting Scientist DDB, NIDDK
	S.A. Wank, R. Vinayek, H. Frucht	Medical Staff Fellows DDB, NIDDK
	L. Miller, H. Stark, I. Waxman	Medical Staff Fellows DDB, NIDDK
	H.C.V. Chiang, J. London, J. O'Brien	Medical Staff Fellows DDB, NIDDK
	L. Zhang, D. Menozzi, T. von Schrenck	Visiting Fellows DDB, NIDDK
<b>COOPERATING UNITS (if any)</b>		
<b>LAB/BRANCH</b> Digestive Diseases Branch		
<b>SECTION</b> Section on Gastroenterology		
<b>INSTITUTE AND LOCATION</b> NIDDK, NIH, Bethesda, Maryland 20892		
<b>TOTAL MAN-YEARS:</b> 5.0	<b>PROFESSIONAL:</b> 3.6	<b>OTHER:</b> 1.6
<b>CHECK APPROPRIATE BOX(ES)</b> <div style="display: flex; justify-content: space-between;"> <div> <input type="checkbox"/> (a) Human subjects  <input type="checkbox"/> (a1) Minors  <input type="checkbox"/> (a2) Interviews         </div> <div> <input checked="" type="checkbox"/> (b) Human tissues         </div> <div> <input type="checkbox"/> (c) Neither         </div> </div>		
<b>SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)</b>  <p>The broad categories which are included in the project are: 1) Characterizing functionally the mechanism by which various substrates cross the plasma membrane of different mammalian cells; 2) identifying the metabolic and humoral factors which influence the transport of various substrates across the plasma membrane; 3) developing techniques which will distinguish between binding of a substrate to the membrane and translocation of the substrate across the membrane; 4) characterizing the mechanism by which the membrane transport of various substrates is altered in certain diseases; and 5) relating these alterations of membrane transport to the pathogenesis and clinical manifestations of the disease.</p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 53002-17 DDB																												
PERIOD COVERED October 1, 1988 to Sept. 30, 1989																														
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Gastrointestinal Hormones																														
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 45%;">J.D. Gardner</td> <td style="width: 20%;">Chief,</td> <td style="width: 20%;">DDB, NIDDK</td> </tr> <tr> <td>Others:</td> <td>R.T. Jensen</td> <td>Chief, Cell Biology Section</td> <td>DDB, NIDDK</td> </tr> <tr> <td></td> <td>P.N. Maton</td> <td>Visiting Scientist</td> <td>DDB, NIDDK</td> </tr> <tr> <td></td> <td>S. Wank, R. Vinayek, H. Frucht</td> <td>Medical Staff Fellows</td> <td>DDB, NIDDK</td> </tr> <tr> <td></td> <td>H. Stark, H-C. V. Chiang, I. Waxman</td> <td>Medical Staff Fellows</td> <td>DDB, NIDDK</td> </tr> <tr> <td></td> <td>S-C. Huang, D. Menozzi, L. Zhang</td> <td>Visiting Fellows</td> <td>DDB, NIDDK</td> </tr> <tr> <td></td> <td>S. Sato</td> <td>Visiting Associate</td> <td>DDB, NIDDK</td> </tr> </table>			PI:	J.D. Gardner	Chief,	DDB, NIDDK	Others:	R.T. Jensen	Chief, Cell Biology Section	DDB, NIDDK		P.N. Maton	Visiting Scientist	DDB, NIDDK		S. Wank, R. Vinayek, H. Frucht	Medical Staff Fellows	DDB, NIDDK		H. Stark, H-C. V. Chiang, I. Waxman	Medical Staff Fellows	DDB, NIDDK		S-C. Huang, D. Menozzi, L. Zhang	Visiting Fellows	DDB, NIDDK		S. Sato	Visiting Associate	DDB, NIDDK
PI:	J.D. Gardner	Chief,	DDB, NIDDK																											
Others:	R.T. Jensen	Chief, Cell Biology Section	DDB, NIDDK																											
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	H. Stark, H-C. V. Chiang, I. Waxman	Medical Staff Fellows	DDB, NIDDK																											
	S-C. Huang, D. Menozzi, L. Zhang	Visiting Fellows	DDB, NIDDK																											
	S. Sato	Visiting Associate	DDB, NIDDK																											
COOPERATING UNITS (if any) Dept. of Chemistry, Case-Western Reserve Univ., Cleveland, Ohio Div. of Cellular Biology, Kennndy Institute for Rheumatology, London, England																														
LAB/BRANCH Digestive Diseases Branch																														
SECTION Section on Gastroenterology																														
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892																														
TOTAL MAN-YEARS: 5.6	PROFESSIONAL: 4.0	OTHER: 1.6																												
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) Human subjects</td> <td><input checked="" type="checkbox"/> (b) Human tissues</td> <td><input type="checkbox"/> (c) Neither</td> </tr> <tr> <td><input type="checkbox"/> (a1) Minors</td> <td></td> <td></td> </tr> <tr> <td><input type="checkbox"/> (a2) Interviews</td> <td></td> <td></td> </tr> </table>			<input type="checkbox"/> (a) Human subjects	<input checked="" type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither	<input type="checkbox"/> (a1) Minors			<input type="checkbox"/> (a2) Interviews																					
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<input type="checkbox"/> (a1) Minors																														
<input type="checkbox"/> (a2) Interviews																														
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>In vitro systems are being used to study the mechanism of action of gastrin, secretin, cholecystokinin, bombesin, substance P and vasoactive intestinal peptide with their specific membrane receptors.</p> <p>Clinical investigators are directed toward developing alternative forms of therapy for and elucidating the pathogenesis of disorders characterized by ectopic production of gastrointestinal hormones (e.g., Zollinger-Ellison syndrome and pancreatic cholera).</p>																														

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 53004-17 DDB

PERIOD COVERED  
October 1, 1988 to Sept. 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Cyclic Nucleotide Mediated Functions

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Jerry D. Gardner	Chief	DDB, NIDDK
Others:	R. T. Jensen	Chief, Cell Biology Section	DDB, NIDDK
	P. N. Maton	Visiting Scientist	DDB, NIDDK
	S. Wank, R. Vinayek, H. Frucht	Medical Staff Fellows	DDB, NIDDK
	L. Zhang, D. Menozzi, J-M. Qian	Visiting Fellows	DDB, NIDDK
	T. von Schrenck, S-C. Huang	Visiting Fellows	DDB, NIDDK
	S. Mantey, C. Sharp	Chemists	DDB, NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH  
Digestive Diseases Branch

SECTION  
Gastroenterology

INSTITUTE AND LOCATION  
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 6.6	PROFESSIONAL: 5.0	OTHER: 1.6
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CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In vitro systems are being used to characterize the mechanism by which cyclic nucleotides alter cell function and to explore the mechanism of action of agents whose effect on cell function is mediated by cellular accumulation of cyclic nucleotides.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 53100- 01DDB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Identification and Characterization of Receptors for GI Peptides		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: R. T. Jensen Chief, Cell Biology Section DDB, NIDDK Others: Jerry D. Gardner Chief, Digestive Diseases Br. DDB, NIDDK Z. Saeed, S. Wank Medical Staff Fellows DDB, NIDDK V. Chiang, H. Frucht Medical Staff Fellows DDB, NIDDK D-H Yu, J-M Qian Visiting Fellows DDB, NIDDK M. Haffar, L-H Wang Visiting Fellows DDB, NIDDK S-C Huang, W. Rowley Visiting Fellows DDB, NIDDK S. Mantey Chemist DDB, NIDDK		
COOPERATING UNITS (if any)		
LAB/BRANCH Digestive Diseases Branch		
SECTION Cell Biology		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 3.71	PROFESSIONAL: 3.71	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p style="margin-left: 40px;">           These studies involve the identification of receptors for gastrointestinal peptides by developing selective radioligands for specific receptors, specific potent antagonists, and the characterization of receptors using these ligands, antagonists, cross-linking studies, solubilization and eventually structural determination.         </p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 53101-01 DDB																								
PERIOD COVERED October 1, 1988 to Sept. 30, 1989																										
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <b>Cellular Basis of Action of Gastrointestinal Peptides</b>																										
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 40%;">PI: R.T. Jensen</td> <td style="width: 40%;">Chief, Cell Biology Section</td> <td style="width: 20%;">DDB, NIDDK</td> </tr> <tr> <td>Others: Jerry D. Gardner</td> <td>Chief</td> <td>DDB, NIDDK</td> </tr> <tr> <td>Z. Saeed, S. Wank</td> <td>Medical Staff Fellows</td> <td>DDB, NIDDK</td> </tr> <tr> <td>V. Chiang, H. Frucht</td> <td>Medical Staff Fellows</td> <td>DDB, NIDDK</td> </tr> <tr> <td>D-H Yu, J-M Qian</td> <td>Visiting Fellows</td> <td>DDB, NIDDK</td> </tr> <tr> <td>M. Haffar, L-H Wang</td> <td>Visiting Fellows</td> <td>DDB, NIDDK</td> </tr> <tr> <td>S-C Huang, W. Rowley</td> <td>Visiting Fellows</td> <td>DDB, NIDDK</td> </tr> <tr> <td>S. Mantey</td> <td>Chemist</td> <td>DDB, NIDDK</td> </tr> </table>			PI: R.T. Jensen	Chief, Cell Biology Section	DDB, NIDDK	Others: Jerry D. Gardner	Chief	DDB, NIDDK	Z. Saeed, S. Wank	Medical Staff Fellows	DDB, NIDDK	V. Chiang, H. Frucht	Medical Staff Fellows	DDB, NIDDK	D-H Yu, J-M Qian	Visiting Fellows	DDB, NIDDK	M. Haffar, L-H Wang	Visiting Fellows	DDB, NIDDK	S-C Huang, W. Rowley	Visiting Fellows	DDB, NIDDK	S. Mantey	Chemist	DDB, NIDDK
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INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892																										
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<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 53501-16 DDB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Studies Relating to the Pathogenesis of Hepatic Encephalopathy		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	E.A. Jones	Chief LDS, NIDDK
Others:	J. Vergalla	Chemist LDS, NIDDK
	S.H. Gammal	Guest Researcher LDS, NIDDK
	M. Lisker-Melman	Visiting Associate LDS, NIDDK
	B.L. Baker	Research Associate LDS, NIDDK
COOPERATING UNITS (if any) Laboratory of Neuroscience, NIDDK (P. Skolnick and A.S. Basile) Laboratory of Molecular Biology, NINCDS (C.D.B. Banner)		
LAB/BRANCH Digestive Diseases Section		
SECTION Liver Diseases Section		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	2.0	1.0
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>The abnormal pattern of visual evoked responses (VERs) in animals with hepatic encephalopathy (HE) due to fulminant hepatic failure (FHF) resembles that associated with encephalopathy induced by drugs which promote GABAergic neurotransmission, including benzodiazepines (BZs). These findings suggest that the pattern of neuronal activity in HE may resemble that associated with activation of the GABA inhibitory neurotransmitter system. Furthermore, rabbits and rats with HE due to FHF exhibit increased resistance to the convulsive effects of bicuculline (a GABA receptor antagonist) and 3-mercaptopropionic acid (an inhibitor of GABA synthesis), respectively. Ameliorations of HE, both clinical and electrophysiologic (VER waveform), have been induced in animals with FHF by BZ receptor antagonists. Furthermore, spontaneous in vitro activity of Purkinje neurons from rabbits in HE due to FHF exhibited increased sensitivity to depression by agonists of the GABA/BZ receptor complex, including a BZ, and, in contrast to control neurons, exhibited excitation when exposed to BZ receptor antagonists. The functional status of the chloride ionophore of the GABA/BZ receptor complex has been shown to be normal in a rat model of HE due to FHF. These findings suggest that in HE due to FHF: (i) There is increased GABA-ergic tone; (ii) Blockading of BZ receptors can ameliorate HE; (iii) BZ receptor antagonists may be of value in the management of HE; and (iv) An endogenous BZ receptor agonist may contribute to HE.</p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 53503-15 DDB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT, (80 characters or less. Title must fit on one line between the borders.) Immunologic Studies of Primary Biliary Cirrhosis		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	E.A. Jones	Chief, LDS, NIDDK
Others:	J.H. Hoofnagle J. Vergalla R. Moreno-Otero	Director, DDDN NIDDK Chemist LDS, NIDDK Guest Researcher LDS, NIDDK
COOPERATING UNITS (if any)  Laboratory of Clinical Investigation, NIAID (S.P. James)		
LAB/BRANCH Digestive Diseases Branch		
SECTION Liver Diseases Section		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 2.0	PROFESSIONAL: 2.0	OTHER:
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>Primary biliary cirrhosis (PBC) appears to be a model autoimmune disease. Abnormal immune mechanisms are being studied in this disease, but so far a disease-specific immunologic deficit has not been defined with certainty. Recently recognized defects of humoral immunity include: (i) Evidence for the existence of an expanded clone of activated B cells that synthesize mitochondrial antibodies with different antigenic specificities from those synthesized by normal B cells; and (ii) Detection of a serum factor, probably an abnormally immunoreactive IgM, which blocks the binding of C3b-opsonized erythrocytes by monocytes. Recently recognized defects in cellular immunity include: (i) A diminished ability of patient T cells to suppress immunoglobulin synthesis; and (ii) Hyporeactivity of lymphocytes in the autologous mixed lymphocyte reaction, which normally leads to activation of suppressor T cells. To determine whether such abnormalities of lymphocyte function in PBC might be due to altered function of immunoregulatory T cell subpopulations, phenotypic and functional characteristics of T cells that have the CD4 antigen detectable (by monoclonal antibody) on their surface were examined. Patients with PBC were found to have normal numbers of CD4+, Leu-8+ T cells, but, in contrast to patients with other liver diseases, suppression of immunoglobulin synthesis and mitogen-stimulated proliferation mediated by this subpopulation of T cells were defective. These defects may play a central role in the abnormal immunoregulation found in this disease.</p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 53505-14 DDB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <b>Studies of Alpha-1-Antitrypsin Phenotypes and Metabolism</b>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	E.A. Jones	Chief LDS, NIDDK
Others:	J. Vergalla	Chemist LDS, NIDDK
	R. Crystal	Medical Officer PB, NHLBI
COOPERATING UNITS (if any)		
LAB/BRANCH Digestive Diseases Branch		
SECTION Liver Diseases Section		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 0.25	PROFESSIONAL: 0	OTHER: 0.25
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)  <p>Protease inhibitor (Pi) phenotypes have been determined using isoelectric focusing on polyacrylamide gel in populations of normal subjects and patients with rheumatoid arthritis, systemic lupus erythematosus, Sjogren's syndrome and hepatocellular carcinoma. The incidence of aberrant (non-MM) phenotypes was not abnormal in any of the four diseases studied, suggesting that alpha-1-antitrypsin deficiency does not play a major role in the pathogenesis of any of these diseases.</p> <p>Using standard tracer methodology to study the turnover of a plasma protein, indices the metabolism of the M and Z alpha-1-antitrypsin molecules have been determined in normal subjects (Pi phenotype MM) and patients with alpha-1-antitrypsin deficiency (Pi phenotype ZZ).</p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 53508-12 DDB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Studies of Hepatic Receptors for Glycoproteins		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	E.A. Jones	Chief  LDS, NIDDK
Others:	J. Vergalla	Chemist  LDS, NIDDK
COOPERATING UNITS (if any)  Laboratory of Biochemistry and Metabolism, NIDDK (G. Ashwell)		
LAB/BRANCH Digestive Diseases Branch		
SECTION Liver Diseases Section		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 0.25	PROFESSIONAL: 0.25	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>             The cellular location and carbohydrate specificities of a glycoprotein recognition system on rat hepatic sinusoidal cells have been determined. The hepatic receptors which recognize N-acetylglucosamine/mannose terminated glycoproteins are located predominantly on endothelial cells. These receptors are glucose sensitive. Fasting increases the number of these receptors whereas diabetes mellitus abolishes this effect of fasting and impairs the function of this receptor <u>in vivo</u>. These findings suggest a mechanism for abnormal glycoprotein metabolism in diabetes mellitus. This carbohydrate recognition system may play an important role in the removal of potentially autodestructive glycoprotein lysosomal hydrolases and other glycoprotein enzymes from the circulation under normal physiological conditions and in disease states.           </p>		

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 53509-11 DDB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of the Natural History and Treatment of Chronic Type B Hepatitis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	E.A. Jones	Chief	LDS, NIDDK
Others:	J.H. Hoofnagle	Director, DDDN	NIDDK
	A. Di Bisceglie	Visiting Scientist	LDS, NIDDK
	M. Lisker-Melman	Visiting Associate	LDS, NIDDK
	P. Martin	Visiting Associate	LDS, NIDDK
	N.V. Bergasa	Medical Staff Fellow	LDS, NIDDK
	J. Korenman	Medical Staff Fellow	LDS, NIDDK
	B.L. Baker	Medical Staff Fellow	LDS, NIDDK

## COOPERATING UNITS (if any)

Georgetown University, Washington, D.C. (J. Gerin)  
 Walter Reed Army Institute of Research, Washington, D.C. (M. Sjogren)

## LAB/BRANCH

Digestive Diseases Branch

## SECTION

Liver Diseases Section

## INSTITUTE AND LOCATION

NIDDK, NTH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

3

## PROFESSIONAL:

2

## OTHER:

1

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A cohort of patients with chronic type B hepatitis is being evaluated and followed to determine the long-term natural history of this common form of chronic liver disease. Selected patients have been entered into therapeutic trials of antiviral and/or immunomodulatory agents. Efforts are now being directed towards improving the therapeutic response rate to interferon alone. Two studies using alpha interferon as therapy for chronic type B hepatitis are underway. The first is a randomized, controlled trial to reevaluate the effects of alpha interferon alone (at a dose of 10 million units three times weekly) compared to no therapy. Twenty three patients have been entered into this study and 11 have now been treated with interferon and completed follow up (total 6 mos.). Among these patients, 5 (45%) cleared HBeAg from serum, 2 had a partial response with loss of DNA polymerase activity from serum but no clearance of HBeAg and the remaining 4 patients showed only a temporary partial inhibition of DNA polymerase activity in serum. The disease in controls did not improve. A second study is designed for patients who have not responded to interferon alone in previous studies. In these cases, the effect of pretreatment with a 4 week course of prednisone before administration of interferon is being evaluated. It is hoped that the immunostimulatory effects of rapid withdrawal of corticosteroids, by inducing an exacerbation of hepatitis activity will tend to optimize the antiviral effects of alpha interferon. Eight patients have been entered into this study so far, and seven have completed the treatment regimen. Only one patient has responded with clearance of HBeAg from serum.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DK 53510-10 DDB
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PERIOD COVERED  
October 1, 1988 to September 30, 1989

TITLE OF PROJECT (40 characters or less. Title must fit on one line between the borders.)  
Studies of the Natural History and Treatment of Chronic Non-A, Non-B (Type C) Hepatitis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	E.A. Jones	Chief	LDS, NIDDK
Others:	J.H. Hoofnagle	Director, DDDN	NIDDK
	A.M. DiBisceglie	Visiting Scientist	LDS, NIDDK
	M. Lisker-Melman	Visiting Associate	LDS, NIDDK
	J. Korenman	Medical Staff Fellow	LDS, NIDDK
	N.V. Bergasa	Medical Staff Fellow	LDS, NIDDK
	B.L. Baker	Medical Staff Fellow	LDS, NIDDK

COOPERATING INSTITUTES (H.J. Alter)  
Division of Digestive Diseases and Nutrition, NIDDK (J. Everhart)  
Armed Forces Institute of Pathology, Washington, D.C. (Z. Goodman)  
Chiron Corporation, Emeryville, CA (M. Houghton, G. Kuo)

LAB/BRANCH  
Digestive Diseases Branch

SECTION  
Liver Diseases Section

INSTITUTE AND LOCATION  
NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 3	PROFESSIONAL: 2	OTHER: 1
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CHECK APPROPRIATE BOX(ES)  
☒ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Patients with well-documented chronic non-A, non-B (NANB) hepatitis are being evaluated to determine the long-term natural history of this common form of chronic liver disease. A cohort of such patients are available to evaluate experimental therapies for this disease. A prospective, randomized, placebo-controlled, double-blind trial of a six month course of human alpha interferon in patients with chronic NANB hepatitis is nearing completion. Forty one patients have completed the first 6 month (blinded) phase of the study. Twenty one received interferon and 20 placebo. Mean serum aminotransferase activities and liver histology improved significantly in interferon-treated patients but not in placebo recipients. Eighteen patients who initially received placebo have been crossed over to receive interferon for 12 months.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 53511-10 DDB
PERIOD COVERED <u>October 1, 1988</u> to <u>September 30, 1989</u>		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <u>Trials of Therapies for Primary Biliary Cirrhosis</u>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	E.A. Jones	Chief LDS, NIDDK
Others:	J.H. Hoofnagle	Director, DDDN NIDDK
	N.V. Bergasa	Medical Staff Fellow LDS, NIDDK
	J. Korenman	Medical Staff Fellow LDS, NIDDK
	T.L. Fong	Medical Staff Fellow LDS, NIDDK
	A. Di Bisceglie	Visiting Scientist LDS, NIDDK
COOPERATING UNITS (if any) <u>Pediatric Services, Universities of Denver and Cincinnati (R.J. Sokol; W.F. Balistreri).</u>		
LAB/BRANCH <u>Digestive Diseases Branch</u>		
SECTION <u>Liver Diseases Section</u>		
INSTITUTE AND LOCATION <u>NIDDK, NTH, Bethesda, Maryland 20892</u>		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
<u>1/2</u>	<u>1/2</u>	
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>Primary biliary cirrhosis (PBC) is a disease of unknown etiology characterized by slowly progressive intrahepatic cholestasis due to non-suppurative, presumably autoimmune, destruction of septal and the larger interlobular bile ducts. Because some other autoimmune diseases appear to respond favorably to certain immunosuppressive agents, trials of selected immunosuppressive drugs are being undertaken in patients with symptomatic PBC. In a randomized controlled trial of chlorambucil therapy, which involved 24 patients, treatment with the drug was associated with a decrease in the rate of increase of serum bilirubin, normalization of elevated serum IgM levels and an improvement in inflammatory cell infiltrate in the liver. These findings strongly suggest that chlorambucil therapy retards the progression of PBC. Unfortunately the potential carcinogenic risk from the administration of chlorambucil, even in small doses, curtails the wider assessment of this drug in PBC. However, the results of the chlorambucil trial indicate that a search for safer and more effective immunosuppressive drugs for PBC is likely to be rewarding. Low-dose methotrexate treatment of this disease is currently being assessed in an open trial.</p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 53514-06 DDB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Immunological Studies in Chronic Viral Hepatitis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	E.A. Jones	Chief	LDS, NIDDK
Others:	J.H. Hoofnagle	Director, DDDN	NIDDK
	A. Di Bisceglie	Visiting Scientist	LDS, NIDDK
	R. Moreno-Otero	Guest Researcher	LDS, NIDDK

COOPERATING UNITS (if any)

Laboratory of Immunoregulation, NIAID (Dr. Julian Ambrus)  
Georgetown University (Dr. Tom Cupps)

LAB/BRANCH

Digestive Diseases Branch

SECTION

Liver Diseases Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

2

PROFESSIONAL:

1.5

OTHER:

.5

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Immunological factors seem to be important in determining the course and outcome of both acute and chronic viral hepatitis. Furthermore, promising therapies for chronic viral hepatitis have profound effects on immune function and sustained responses to therapy may depend largely on restoration of normal immune responsiveness. The role of immunologic mechanisms in determining the course and ultimate outcome of viral hepatitis is being studied and the effects of therapies on the immune system are being evaluated. Serial studies of cellular immune function have been conducted on patients with chronic type B hepatitis.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 53515-03 DDB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Studies of the Natural History and Treatment of Duck Hepatitis B Virus Infection		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	E.A. Jones	Chief  LDS, NIDDK
Others:	J.H. Hoofnagle P. Martin B.L. Baker	Director, DDDN Visiting Associate Medical Staff Fellow  NIDDK LDS, NIDDK LDS, NIDDK
COOPERATING UNITS (if any) Comparative Animal Unit (H. Robcis) Hepatitis Virus Section, NIAID (R. Miller) Clinical Oncology Program, NCI (H.Mitsuya, S. Broder)		
LAB/BRANCH Digestive Diseases Branch		
SECTION Liver Diseases Section		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	2	PROFESSIONAL: 1.75 OTHER: 0.25
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>There are many similarities in structure and properties between the human hepatitis B virus (HBV) and the duck hepatitis B virus (DHBV). These similarities suggest that DHBV infection in ducks may be a useful experimental model of human HBV infection, particularly as HBV cannot be grown readily in cell culture. Ducks infected with DHBV at birth become chronic carriers of the virus, although they may not develop overt hepatitis. Some DHBV-infected ducks have been reported to develop hepatocellular carcinoma. The analogous human tumor is strongly linked etiologically with chronic hepatitis B infection. As a screening test for new effective therapies for chronic type B hepatitis in man, new antiviral and immunomodulatory agents are being assessed for their ability to suppress DHBV replication in ducks.</p>		

ANNUAL REPORT OF THE  
MOLECULAR, CELLULAR AND NUTRITIONAL ENDOCRINOLOGY BRANCH  
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The MCNEB continues basic and clinical investigations in the areas of molecular regulation and neuroendocrinology (Molecular Regulation and Neuroendocrinology Section, Bruce D. Weintraub, Chief); experimental diabetes, metabolism and nutrition (Experimental Diabetes, Metabolism and Nutrition Section, Samuel W. Cushman, Chief); and growth and development (Growth and Development Section, Matthew M. Rechler, Chief). The Branch has had many visiting fellows and associates, as well as international collaborations with the University of Milan, Italy; University of Marseilles, France; Karolinska Institute, Sweden; Postgraduate School of Obstetrics and Gynaecology, University of Auckland, New Zealand; University of Naples, Italy; Department of Medicine, University of Gothenburg, Sweden; Endocrine Institute, Rambam Medical Center, Haifa, Israel; Department of Biochemistry, the University of Newcastle upon Tyne, England.

Dr. Weintraub was honored as the Farahe Maloof Lecturer at the Massachusetts General Hospital and the Bly Stein Visiting Professor at the University of Southern California Medical School.

I. GLYCOPROTEIN HORMONES: MOLECULAR BIOLOGY, SYNTHESIS, PROCESSING REGULATION, ACTION AND PATHOPHYSIOLOGY

A. Primary Hypothyroidism

We have previously reported changes in thyrotropin (TSH) carbohydrate structure during ontogenesis. We have now studied the effect and specificity of congenital hypothyroidism on rat TSH carbohydrate structure versus free- $\alpha$  subunit and total proteins. Secreted TSH and free- $\alpha$  from rat pituitaries labeled with  $^3\text{H}$ -glucosamine were precipitated with specific antisera and total proteins were acid precipitated. Labeled carbohydrate chains were released by endoglycosidase F and analyzed by anion-exchange HPLC. TSH from thyroidectomized animals contained more oligosaccharides with  $\geq 3$  negative charges compared to controls ( $p < .01$ ), while those from free- $\alpha$  and total proteins were unchanged. Also, thyroidectomized animals had a greater ratio of sialylated (N) to sulfated (S) species compared to controls for oligosaccharides containing both 1(N1/S1) and 2(N2/S2) negative charges. For TSH- $\alpha$ , N1/S1 and N2/S2 were increased 2.9 and 7.4-fold in thyroidectomized compared to control animals ( $p < .01$ ); for TSH- $\beta$  these increases were 15.1 and 25.5-fold, respectively ( $p < .01$ ). There was no change in these ratios for total proteins and only a small change in free- $\alpha$ . In conclusion, primary hypothyroidism produces specific changes in secreted TSH consisting of more negatively charged oligosaccharides with increased sialylation.

We have also investigated the effect of *in vivo* hypothyroidism on the sialylation and sulfation of thyroid-stimulating hormone (TSH) secreted by mouse pituitary explants. Oligosaccharides from secreted thyroid-stimulating hormone from hypothyroid animals contained greater sialic acid relative to sulfate in both  $\alpha$  and  $\beta$  subunits. Aging *per se* had little effect on thyroid-

stimulating hormone sialylation or sulfation. Variable sialylation and sulfation demonstrates a mechanism demonstrates a mechanism for charge microheterogeneity of thyroid-stimulating hormone, and the increasing sialylation observed with hypothyroidism may functionally mediate the prolonged metabolic clearance that has been noted previously.

. . . . P. W. Gyves, B. D. Weintraub

#### B. Central Hypothyroidism.

To determine the role of hypothalamic TRH *in vivo* in regulating structural characteristics of TSH carbohydrate chains, adult rats received paraventricular nuclear (PVN) lesions (N=6) or sham lesions (N=6). The paraventricular nuclei contain large amounts of TRH and rats with lesions in these hypothalamic nuclei have been shown to have decreased plasma thyroid hormone levels. at 10 d after surgery, SC osmotic pumps infusing saline or 1 mg/kg/d TRH were placed. At 14 d after surgery, pituitaries were removed and incubated with  $^3\text{H}$ -glucosamine for 24 h. Glycopeptides prepared from secreted TSH were sequentially eluted from Concanavalin-A chromatography selecting unbound, weakly bound, and strongly bound forms.

Plasma free T4 was lower in the PVN lesioned rats treated with saline than sham lesioned rats treated with saline ( $1.6 \pm 0.4$  vs.  $5.2 \pm 0.1$  ng/dl,  $p < 0.001$ ). *In vivo* TRH administration in the PVN lesioned group normalized plasma free T4 but had no effect on free T4 in the sham group. Secreted TSH glycopeptides in the PVN lesioned rats treated with saline as compared to sham lesioned rats treated with saline had fewer unbound forms reflecting multiantennary structures ( $43 \pm 4$  vs.  $57 \pm 1\%$ ;  $p < 0.05$ ) and more weakly bound forms reflecting biantennary structures ( $50 \pm 4$  vs.  $35 \pm 2\%$ ;  $p < 0.05$ ). TRH administration *in vivo* normalized the Concanavalin-A binding pattern of secreted TSH glycopeptides in the PVN lesioned group but had no significant effect in the sham lesioned group. TSH  $\alpha$  subunit demonstrated both multi- and biantennary forms, but TSH- $\beta$  subunit showed a predominance of multiantennary forms in both the PVN and sham lesioned groups treated with saline. *In vivo* changes in TRH levels altered TSH carbohydrate characteristics as described above for both subunits.

In summary, hypothalamic hypothyroidism altered TSH carbohydrate structures and *in vivo* TRH administration normalized these structures in parallel with the correction of serum free T4. In addition to reported quantitative changes in TSH in response to thyroid hormone, these qualitative changes may have an important effect on TSH action.

. . . . T. Taylor, B. D. Weintraub

#### C. Role of Carbohydrate in the Action of TSH

Enzymatic deglycosylation of bovine TSH and  $\alpha$  and  $\beta$  subunits was performed by Endo-glycosidase F digestion of the subunits and purification of the products on concanavalin A affinity chromatography to remove the released oligosaccharides and the intact undigested protein from the deglycosylated material. Carbohydrate compositional analysis of the deglycosylated subunits was performed using Dionex HPLC system with the ultrasensitive Pulsed Amperometric Detection method for monosaccharides. One residue of glucosamine per glycan chain was found remaining on the protein and all other monosaccharides were completely removed by the above procedure.

The deglycosylated subunits thus prepared by recombined to obtain deglycosylated TSH, which migrated as a 34 kD band on SDS-PAGE as compared to the native at 40 kD position. Receptor binding activity of the deglycosylated bTSH was not altered when measured in an assay using bovine thyroid membranes. In an *in vitro* bioassay using stimulation of cyclic AMP production in FRTL-5 cells, deglycosylated bTSH showed reduced activity with a potency of five to ten-fold lower than that of control, although the  $V_{\max}$  remained unaltered. In contrast, the deglycosylated bTSH showed a reduction in  $V_{\max}$  when assayed for its adenylyl cyclase stimulatory activity in bovine thyroid membranes. These results indicate that complete deglycosylation can be achieved by Endoglycosidase F digestion and recombination of the subunits and that enzymatically deglycosylated TSH showed a decrease in potency in two assays but a decreased  $V_{\max}$  in only one assay. Deglycosylated bTSH was also prepared by Endoglycosidase F digestion of intact b TSH and purifying the completely deglycosylated product on concanavalin A affinity column. This preparation also showed properties identical to the one prepared by subunit recombination. Previous reports using chemical methods have apparently overestimated the effects of deglycosylation, while those using endoglycosidases have apparently underestimated these effects.

. . . . N. R. Thotakura, B. D. Weintraub

#### D. Effects of Glycopeptides and Oligosaccharides on hCG-receptor Interactions

Deglycosylation of gonadotropins and thyrotropin results in a major loss of hormonal bioactivity, while not impairing receptor binding activity. However, a direct role of the glycan moieties in hormonal signal transduction and adenylyl cyclase activation has not been demonstrated. The addition of carbohydrate chains together with the deglycosylated hormone does not restore the hormonal activity. In contrast, glycopeptides were found to inhibit hCG-stimulated adenylyl cyclase activity and hCG binding to its receptor. In the present study, glycopeptides and oligosaccharides prepared from hCG, transferrin (both biantennary complex), fetuin (triantennary complex),  $\alpha_1$  acid glycoprotein (tetraantennary complex) and ovalbumin (high mannose) have been shown to inhibit the binding of hCG to its receptor. The inhibition was also observed with a highly purified preparation of the receptor, thus suggesting a lack of involvement of other lectin-like membrane components as others have previously proposed. individual monosaccharides and peptides without any oligosaccharide chains did not inhibit hCG binding to receptor, indicating that the effect was specific to a common oligosaccharide core present in all of these glycopeptides and oligosaccharides. Adenylyl cyclase activity stimulated by hCG, isoproterenol or forskolin was inhibited by oligosaccharides, indicating a non-specific interaction. The oligosaccharides did not affect the *in vitro* bioactivities of hCG and bTSH tested in cultured cells. These results suggest that Asn-linked oligosaccharide chains from various glycoproteins nonspecifically perturb hCG-receptor interactions through a putative carbohydrate binding site on the receptor.

. . . . N. R. Thotakura, B. D. Weintraub

#### E. Increased Bioactivity of Thyrotropin from Human Thyrotropic tumors

We have previously reported a patient with a TSH-producing tumor whose serum TSH showed increased bioactivity in an adenylyl cyclase assay. In the last year we have examined the

bioactivity of serum TSH from several patients with thyrotropic tumors and found increased bioactivity in a cyclic AMP assay using rat FRTL thyroid cells. After resection of the tumor in several patients, the TSH activity was restored to normal. These data suggest that the hyperthyroidism resulting from such tumors may be, in part, related to qualitative changes in the molecule, probably resulting from altered glycosylation.

... N. Gesundheit, B. D. Weintraub

#### F. Regulation of Human TSH- $\beta$ Gene Transcription by Thyroid Hormone.

We have localized in the first exon of the human TSH- $\beta$  gene a region that stimulates gene expression in the absence of L-T<sub>3</sub> and mediates a concentration-dependent decrease in expression in the presence of L-T<sub>3</sub>. Half-maximal suppression was observed at approximately  $5 \times 10^{11}$  M L-T<sub>3</sub> in serum-free conditions. DNA sequences from +3 to +37 bp conferred L-T<sub>3</sub> inhibition to heterologous promoter. This region also binds a nuclear thyroid hormone receptor, c-erbA $\beta$ , indicating that it fulfills criteria for a thyroid hormone inhibitory element.

In 293 cells, DNA sequences between +9 and +37 enhanced gene expression from pTSH $\beta$ CAT constructs as much as 6-fold, as assessed by CAT activity. These data indicate that, in the absence of thyroid hormone, this region was important in stimulating basal activity of the human TSH- $\beta$  promoter. Primer extension data clearly indicate that this was a pretranslational effect and strongly suggest that this region was necessary for accurate transcription initiation since we detected only aberrant transcription initiation without this element.

... F. E. Wondisford, J. McClaskey, B. D. Weintraub

#### G. Regulation of Human TSH- $\beta$ Gene Transcription by Thyrotropin-Releasing Hormone and cAMP.

The expression of the human thyrotropin  $\beta$ -subunit gene (TSH- $\beta$ ) as well as its regulation by TRH and cAMP was examined in a clonal pituitary cell line, GH<sub>3</sub>. Transient expression studies were performed with chimeric plasmids containing different lengths of 5' flanking human TSH- $\beta$  DNA coupled to the reporter gene, chloramphenicol acetyltransferase (CAT). The constructs contained -1200 to +8 bp, -1200/+8 bp with an internal deletion from -613 to -199 bp, and -128/+8 bp of the human TSH- $\beta$  gene. The construct with the internal deletion from -613 to -199 bp was expressed with a higher efficiency than the -1200/+8 bp construct suggesting the presence of sequences mediating tissue specific repression in the region from -613 to -199 bp.

The regulation of the expression of these plasmids by cAMP was tested with forskolin, a drug that directly activates the catalytic subunit of adenylate cyclase. Forskolin (10  $\mu$ M) produced a 6-10 fold induction of CAT activity with all three constructs, while TRH treatment (10 nM) resulted in a 2-4 fold stimulation. The proportion of the effects of the agents was similar with all three constructs, forskolin being consistently 2-3 more potent than TRH. The concentration response to both agents was tested with the shortest construct (-128/+8 bp). Forskolin induced a 4-11 fold induction in the range tested (0.32-25  $\mu$ M), the maximal effect being observed at 10  $\mu$ M. TRH (0.1-100 nM) produced a 2.5 fold increase, the maximal effect being achieved at 10 nM.

The time course of the effects of these agents was measured with maximally effective concentrations over a period of 72 h. The maximal induction by forskolin (10  $\mu$ M) was observed after 8 h, whereas basal expression and expression in TRH-treated cells was maximal after 24-48 h.

The induction observed with the combined maximally effective concentrations of forskolin (10  $\mu$ M) and TRH (10 nM) was more than additive suggesting different modes of action. Both agents together resulted in a 23-fold stimulation of CAT activity compared to induction ratios for the single agents of 10.5 (forskolin) and 3.4 fold (TRH). The forskolin effect seemed to be mediated by cAMP since dideoxyforskolin, a derivative that has no effect on adenylate cyclase, did not induce CAT activity. Furthermore, cAMP analogues like 8-Br-cAMP and 8-chlorophenylthio-cAMP and the phosphodiesterase inhibitor, IBMX, were also capable of inducing CAT activity when tested for their effects on the expression of the -128/+8 bp construct.

. . . H. J. Steinfelder, F. E. Wondisford, B. D. Weintraub

#### H. Site-Directed Mutagenesis of the hTSH $\beta$ Gene.

Prior studies probing the structure-function correlates of pituitary glycoproteins have provided insight into the importance of certain subunit regions in hormone assembly and function. However, many of these studies have used chemical modifications that may have had other, unintended effects on these hormones. Thus, a process that resulted in specific amino acid changes in the  $\beta$  subunit might provide new insights into glycoprotein structure and function.

We initially made mutations in the glycosylation recognition sequence of the hTSH- $\beta$  subunit, and produced normal and mutant recombinant TSH through transient transfection techniques developed in our laboratory. We found that mutations that prevent glycosylation result in a greater than 93% reduction in TSH secretion. Mutant  $\beta$  subunit production was also decreased compared to normal  $\beta$  subunit. Another mutation in this region maintained glycosylation, but still resulted in a 70% decrease in TSH secretion.

This latter result suggested that changes in the glycosylation recognition sequence might have effects apart from that of inhibiting glycosylation. We noted that this sequence is near a region common to nearly all of the glycoprotein hormone  $\beta$  subunits, the CAGYC region. We suspected that changes near the CAGYC region might be interfering with hormone synthesis. We tested this hypothesis by duplicating a known human mutation within the CAGYC region associated with a complete deficiency of TSH. Our duplication of this mutation also resulted in the complete absence of TSH production.

We have also examined the carboxyterminal region of the hTSH- $\beta$  subunit. The amino acid sequence based on peptide analysis predicts a protein of 112 amino acids. However, the gene sequence predicts 118 amino acids. This difference might be due to degradation during protein purification, or could be the result of posttranslational processing. To determine if the 112 amino acid form could assemble into TSH, we created a mutant  $\beta$  subunit with a termination codon at codon 113. This 112 amino acid form of hTSH- $\beta$  subunit was secreted in significantly greater amounts than TSH with a  $\beta$  subunit initially translated as 118 amino acids.

Experiments are now in progress to define further structure-function relationships of CAGYC region and the carboxyterminus. In addition, receptor binding and bioactivity studies of mutant TSH are in progress.

. . . R. W. Lash, B. D. Weintraub

#### I. Development of Thyroid Hormone Regulation of TRN mRNA Levels in rat Paraventricular Nuclei During Ontogeny.

The changing role of the hypothalamus and pituitary in regulating thyroid hormone levels in the rat during ontogeny has not been fully elucidated. It has been reported that TRH begins to stimulate TSH secretion at 5 to 8 days after birth but that the pituitary responds to hypothyroidism during late gestation. To determine the onset and extent of TRH response to low thyroid hormone levels during ontogeny, normal, and hypothyroid rats treated with methimazole for 7 days, were sacrificed at 16 days gestation (16g), 20 days gestation (20g), 7 days, 21 days, and 56 days after birth. Plasma hormones were assayed from pregnant mothers, pups and adults. TRH mRNA was measured in the PVN of brain slices by the sensitive and anatomically specific method of in situ hybridization histochemistry using a labeled 48 base cDNA oligonucleotide for TRH and quantitation was performed by digitized computer analysis.

Plasma free  $T_4$  was decreased after methimazole treatment as compared to age matched controls at 20g (0.2 vs 0.7 ng/ml), 7 days (<0.1 vs 0.4 ng/ml), 21 days (<0.1 vs 0.9 ng/ml), and 56 days (0.2 vs 1.3 ng/ml). Plasma TSH increased after methimazole treatment as compared to age matched controls at 20g (6.6 vs 2.8 ng/ml), 7 days (13.9 vs 1.3 ng/ml), 21 days (17.0 vs 1.6 ng/ml), and 56 days (8.1 vs 1.1 ng/ml). TRH mRNA was detected in the PVN at 16g after brain slices were dipped in emulsion. Basal TRH mRNA in the PVN increased with age until 7 days then plateaued. Hypothyroid rats as compared to age match controls had TRH mRNA levels of 110% at 20g ( $166 \pm 5$  vs  $150 \pm 9$  OD units; NS) 121 % at 7 days ( $269 \pm 9$  vs  $222 \pm 5$  OD units;  $p < 0.001$ ), 176% at 21 days ( $461 \pm 26$  vs  $252 \pm 27$  OD units;  $p < 0.001$ ), and 226% at 56 days ( $545 \pm 20$  vs  $244 \pm 6$  OD units;  $p < 0.001$ ).

Thus, TRH is present at 16 days gestation, increases with maturation, but does not respond to altered thyroid hormone levels until 7 days after birth. In conclusion, we have shown that the onset of TRH response to thyroid hormone feedback corresponds to the time when TRH begins to regulate TSH secretion and that the increase in fetal plasma TSH observed in late gestation is independent of hypothalamic TRH regulation.

. . . . T. Taylor

#### J. Molecular Abnormalities of the c-erbA $\beta$ gene in Generalized Thyroid Hormone Resistance

Generalized thyroid hormone resistance (GTHR) is a disorder of thyroid hormone action characterized by elevated free thyroid hormones and TSH, and inappropriate clinical and biochemical signs of euthyroidism or hypothyroidism. It is usually transmitted as a familial syndrome with an autosomal dominant pattern of inheritance. We have previously shown that in a kindred, the gene for GTHR is tightly linked to one of the two known thyroid hormone receptor genes, c-erbA $\beta$ , on chromosome 3. To study the role of the thyroid hormone receptor genes in this

syndrome, we have conducted further linkage analyses in two other kindreds, B and D, with differing degrees of thyroid hormone insensitivity. There was also linkage between c-erbA $\beta$  and GTHR loci in these two kindreds, and the combined maximum lod score for all three kindreds at a recombination fraction of 0 was 5.77. We investigated the defect in c-erbA $\beta$  in Kindred A by sequencing portions of the T<sub>3</sub>-binding domain in the 3'-region of fibroblast c-erbA $\beta$  cDNA and leukocyte c-erbA $\beta$  genomic DNA. A base substitution, cytosine to adenine, was found at cDNA position 1643 which altered the proline codon to a histidine codon. This base substitution was only found in one allele in Kindred A, as expected for a dominant disease, and was not found in Kindreds B or D, or in 92 random c-erbA $\beta$  alleles. Because of this absolute linkage with the abnormal phenotype, and the fact that the mutation is predicted to alter the secondary structure of the crucial T<sub>3</sub>-binding domain of the c-erbA $\beta$  receptor, it is probably the c-erbA $\beta$  mutation responsible for GTHR in kindred A. Different mutations in the c-erbA $\beta$  gene are likely responsible for the variant phenotypes of thyroid hormone resistance in Kindreds B and D.

... S. J. Usala, B. D. Weintraub

## II. HORMONES AND RECEPTORS

### A. Insulin-like Growth Factors

We have continued our studies of the insulin-like growth factors (IGFs), their receptors and binding proteins to understand the role of the IGFs in physiological and pathological growth. During the past year we have demonstrated that: (1) multiple rat IGF-II mRNAs (1.0 to 4.67 kb) arise from a single gene through the use of 2 promoters and 2 polyA addition sites; (2) the IGF-II gene is transcribed from both promoters in multiple non-neural tissues in fetal rats and in fetal and adult rat brain; (3) IGF-II mRNA is expressed at high levels by a human neuroblastoma cell line, and by human neuroblastoma and pheochromocytoma tumors; (4) IGF-II synthesized by this human neuroblastoma cell line stimulates autonomous growth via the IGF-I receptor; (5) IGF-II inhibits the binding of a lysosomal enzyme,  $\beta$ -galactosidase to the IGF-II/Mannose 6-phosphate receptor, and may modulate the distribution and function of lysosomal enzymes; (6) the IGF-II/Man 6-P receptor is present at high levels in fetal rat tissues and in serum from fetal rats and monkeys, and decreases with developmental age; (7) the nucleotide sequence of an IGF-binding protein, BP-3A, indicates that it is one of a family of related proteins; (8) a single BP-3A mRNA of ~2kb is expressed in multiple fetal rat tissues and adult rat brain; (9) a carboxyl-terminal fragment of BP-3A retains the ability to bind IGF-I and IGF-II, suggesting that it contains at least part of the IGF binding domain; (10) BP-3A is the predominant IGF binding protein in fetal rat serum; (11) the major 150 kDa IGF binding protein complex in adult rat serum consists of a glycosylated IGF-binding subunit (BP-53) that associates with a nonbinding subunit in the presence and absence of IGFs; and (12) rat BP-53 contains two IGF-binding sites (on the same or different molecules) whose specificity is unaffected by enzymatic deglycosylation.

... M. M. Rechler, A. L. Brown, D. E. Graham, C. C. Orlowski, Y. W.-H. Yang, J. A. Romanus, L. Tseng, G. T. Ooi

### III. STUDIES OF THE MECHANISM OF THE INSULIN ACTION AND ITS PERTURBATION IN ALTERED STATES

#### A. Insulin-Cell Interaction

The phosphorylation state and tyrosine kinase activity of insulin receptors in subfractions of insulin-treated rat adipose cells have been studied. The results suggest that insulin receptors retain their kinase activity on internalization, indicating that the receptor kinase may possibly mediate insulin's effects while inside the cell. However, if the internalized receptor kinase mediates insulin's effect on glucose transport, only a portion of its maximum activity appears to be necessary for full transport stimulation (<20%). Further, the difference in kinase activity among subfractions suggests that the receptor kinase in the low-density microsomes may be in the process of deactivation. The effects of isoproterenol and insulin on the subcellular distribution and phosphorylation state of insulin receptors have been investigated in rat adipose cells. The results suggest that isoproterenol augments insulin's effect on receptor internalization, but reverses its stimulatory effect on receptor phosphorylation state and tyrosine kinase activity in plasma membranes; the latter effects may account for the decreased sensitivity of the glucose transport response to insulin.

. . . . T. M. Weber, I. A. Simpson, S. W. Cushman

#### B. Insulin's Regulation of Glucose Transport

Insulin's rapid action to increase glucose transport is believed to occur primarily through the translocation of glucose transporters from an intracellular pool to the plasma membrane. To better understand the mechanism involved, we studied the role of protein synthesis in glucose transporter translocation by using the protein synthesis inhibitor, cycloheximide. The results suggest that insulin's stimulation of glucose transport and translocation of glucose transporters can occur without acute protein synthesis. The possible role of protein kinase C in the regulation of glucose transport in the rat adipose cell has been examined. While the results do not clarify the relationship between protein kinase C and the mechanism of insulin action, they do suggest that 1) protein kinase C activation induces the translocation of glucose transporters without a corresponding increase in glucose transport activity and 2) insulin and PMA appear to stimulate glucose transport in rat adipose cells through distinct but interactive mechanisms. In another separate study, the data suggest that 1) glucose transporters redistributed in response to PMA or vasopressin exhibit different intrinsic activities compared to glucose transporters from insulin-stimulated cells and 2) the phosphorylation state of glucose transporters may regulate their activity. The question of a long-term regulatory role of insulin on adipocyte glucose transporter content has been addressed using the differentiating or fully mature 3T3-F442A adipocytes. The results suggest that insulin plays a long-term regulatory role on cultured adipocyte glucose transporter content through a selective effect on the erythrocyte/HepG2/brain-type glucose transporter. A procedure for purification of the 45 kDa transport protein from rat brain has been developed. An  $\approx 5,000$ -fold purification of the rat brain glucose transporter has been achieved with a yield of 25%.

. . . . S. W. Cushman, I. A. Simpson, T. M. Weber, M. J. Zarnowski, D. R. Yver, A. D. Habberfield, T. L. Jones, J. Saltis, S. Krief, K. Chundu, T. Davies, O. Gonzalez-Mulero, C. Londos, J. J. Egan

### C. Alterations in Insulin's Action in Insulin-Dependent Diabetes Mellitus

Diabetes and its treatment with insulin in the rat result in dramatic changes in insulin-stimulated glucose transport activity and glucose transporter number in adipose cells. In addition, at least two genetically distinct glucose transporters coexist in adipose cells, one cloned from human hepatoma cells and rat brain (hep G2/brain) and another from rat skeletal muscle, heart and adipose cells (adipose cell/muscle). Here we demonstrate differential regulation of these two glucose transporters in adipose cells of diabetic and insulin-treated diabetic rats and compare changes in the expression of each glucose transporter with marked alterations in insulin-stimulated glucose transport activity. The results suggest that 1) altered expression of the adipose cell-muscle glucose transporter forms the molecular basis for the dysregulated glucose transport response to insulin characteristic of diabetes, 2) the expression of two types of glucose transporters in rat adipose cells is regulated independently, and 3) alterations in mRNA levels are only part of the mechanism for an *in vivo* regulation of the expression of either glucose transporter. Evidence has recently accumulated for a direct role of glucose, independent of insulin, in the regulation of cellular glucose transport. Moreover, we have demonstrated the reversal of *in vivo* insulin resistance in diabetic rats by normalization of hyperglycemia without any change in plasma insulin concentration. In the present study, the effect of correction of hyperglycemia on insulin's stimulatory action on glucose transport activity in adipose cells from diabetic rats has been examined. The data show that normalization of the plasma glucose concentration in the absence of insulin therapy in diabetic rats restores, or may even enhance, the *in vitro* adipose cell glucose transport response to insulin while normalizing *in vivo* insulin-mediated glucose disposal and suggest that the plasma glucose concentration is an important regulator of glucose transport activity in adipose cells, independent of the plasma insulin concentration.

... S. W. Cushman

### D. Insulin's Regulation of Hormone Binding

A comparison of insulin's effects on glucose transport and cell surface IGF-II receptors has been undertaken in rat adipose cells using 3-O-methylglucose transport as a measure of glucose transport activity and Scatchard analysis of IGF-II binding in the presence of KCN to determine cell surface IGF-II receptor number. The results demonstrate that while the characteristics of the stimulatory action of insulin on glucose transport activity and cell surface IGF-II receptor number are qualitatively similar, quantitative differences are clearly demonstrable which suggest that the subcellular cycling of these two integral membrane proteins occurs by distinct processes. The effects of adenosine, isoproterenol, and glucose have now been examined on both steady state insulin responsiveness and sensitivity in this cell type prepared in the presence of saturating adenosine (200 nM). The results show that the stimulatory effect of insulin on IGF-II binding to rat adipose cells is modulated not only by counterregulatory hormones, but also by glucose, a major substrate of insulin action.

... S. W. Cushman, I. A. Simpson

#### E. Counterregulation of Insulin's action by Catecholamines

Insulin shifts the steady state subcellular distribution of IGF-II receptors from a large intracellular pool to the plasma membrane in the rat adipose cell. In the present study, the counterregulatory effects of adrenergic stimulation, adenosine deaminase (ADA), and cAMP on this process have been studied. The results suggest that  $\beta$ -adrenergic stimulation, through a cAMP-dependent mechanism, markedly alters the insulin-stimulated redistribution of IGF-II receptors. This effect is additional to the potent antagonistic action of cAMP on insulin's signaling mechanism. We have also examined the modulation of protein kinase C-mediated stimulation of glucose transport activity in the rat adipose cell by 1) ligands for receptors that mediate stimulation ( $R_s$ ; lipolytic) or inhibition ( $R_i$ ; antilipolytic) of adenylate cyclase and 2) pertussis and cholera toxins which regulate the inhibitory ( $G_i$ ) and stimulatory ( $G_s$ ) guanyl nucleotide-binding proteins of adenylate cyclase, respectively. The results suggest that 1) G-proteins modulate protein kinase C-mediated stimulation of glucose transport activity by altering the intrinsic activity of glucose transporters residing in the plasma membrane and 2) a functional  $G_i$ -protein is required for stimulation of glucose transport activity by phorbol esters and vasopressin.

... I. A. Simpson J. Saltis, A. D. Habberfield, H. Nishimura, S. W. Cushman,  
M. J. Zarnowski, C. Londos.

#### F. Alterations in Insulin's Action with Fasting/Refeeding

The effects of fasting and refeeding on the glucose transport response to insulin in isolated rat adipose cells have been examined. The results suggest that the insulin-resistant glucose transport in isolated adipose cells from fasted rats can be explained by a decreased translocation of glucose transporters to the plasma membrane due to a depleted intracellular pool. In contrast, the insulin hyperresponsive glucose transport observed with refeeding appears to result from 1) a restored translocation of glucose transporters to the plasma membrane from an intracellular pool replenished through an increase in intracellular protein and 2) enhanced plasma membrane glucose transporter intrinsic activity. The human Hep G2 glucose transporter cDNA clone has been used to examine the molecular basis for these alterations. The data suggest that the abundance of mRNAs for multiple adipose cell genes is affected by fasting and refeeding. In particular, this is the first demonstration in an insulin-sensitive tissue that glucose transporter number, and hence a major factor in the glucose transport response to insulin, may be controlled, at least in part, by alterations in mRNA abundance. Insulin increases glucose transport activity and IGF-II binding in rat adipose cells by eliciting the redistribution of glucose transporters and IGF-II receptors from large intracellular pools to the plasma membrane. We now have measured cell surface IGF-II binding in intact cells from 2-day fasted and 2-day fasted/6-day refed rats and assessed IGF-II receptor number in subcellular membrane fractions by immunoblotting. The results suggest that fasting differentially regulates the number and distribution of IGF-II receptors and glucose transporters in adipose cells. This finding suggests distinct intracellular trafficking pathways for these proteins. Nutritional regulation of the IGF-II receptor may serve as a tool to explore the physiological role of IGF-II.

... S. W. Cushman, I. A. Simpson

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER <b>ZO1 DK 55000-17MCNE</b>																
PERIOD COVERED <b>October 1, 1988 to September 30, 1989</b>																		
TITLE OF PROJECT (80 characters or less. This must fit on one line between the borders.) <b>Biosynthesis and Glycosylation of Thyrotropin</b>																		
PRINCIPLE INVESTIGATOR (List other professional personnel below the Principle Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 15%; vertical-align: top;">PI:</td> <td style="width: 30%;">B. D. Weintraub</td> <td style="width: 30%;">Chief</td> <td style="width: 25%;">MCNEB, NIDDK</td> </tr> <tr> <td style="vertical-align: top;">OTHERS:</td> <td>P. W. Gyves</td> <td>Senior Staff Fellow</td> <td>MCNEB, NIDDK</td> </tr> <tr> <td></td> <td>T. Taylor</td> <td>Guest Researcher</td> <td>MCNEB, NIDDK</td> </tr> <tr> <td></td> <td>G. S. DeCherney</td> <td>Guest Researcher</td> <td>MCNEB, NIDDK</td> </tr> </table>			PI:	B. D. Weintraub	Chief	MCNEB, NIDDK	OTHERS:	P. W. Gyves	Senior Staff Fellow	MCNEB, NIDDK		T. Taylor	Guest Researcher	MCNEB, NIDDK		G. S. DeCherney	Guest Researcher	MCNEB, NIDDK
PI:	B. D. Weintraub	Chief	MCNEB, NIDDK															
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	T. Taylor	Guest Researcher	MCNEB, NIDDK															
	G. S. DeCherney	Guest Researcher	MCNEB, NIDDK															
COOPERATING UNITS (if any) None																		
LAB/BRANCH Molecular, Cellular and Nutritional Endocrinology Branch																		
SECTION Molecular Regulation and Neuroendocrinology Section																		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892																		
TOTAL MAN-YEARS: 2.5	PROFESSIONAL: 2.5	OTHER: 0																
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews																		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>             We have been intrested in the endocrine regulation of thyrotropin (TSH) carbohydrate structure and the physiologic effects of such qualitative changes. Using newly developed methods of high performance liquid chromatography and lectin analysis of labeled carbohydrate chains, we have shown that TSH carbohydrate is more sialylated and banded in rats with primary hypothyroidism. This effect is most dramatic in neonatal hypothyroidism. Paraventricular lesions of the hypothalamus causing central hypothyroidism result in TSH with less banding, which can be restored to normal by injection of thyrotropin-releasing hormone. These changes in carbohydrate structure appear to change TSH clearance and action <u>in vivo</u>.           </p>																		

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. This must fit on one line between the borders.)

Regulation and Action of Thyrotropin

## PRINCIPLE INVESTIGATOR (List other professional personnel below the Principle Investigator.) (Name, title, laboratory, and institute affiliation)

PI: B. D. Weintraub Chief MCNEB, NIDDK

OTHERS: N. R. Thotakura Visiting Associate MCNEB, NIDDK  
N. Gesundheit Senior Staff Fellow MCNEB, NIDDK

## COOPERATING UNITS (if any)

None

## LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

## SECTION

Molecular Regulation and Neuroendocrinology Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.2

## PROFESSIONAL:

1.2

## OTHER:

0

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The role of carbohydrate in the action of thyrotropin (TSH) and other glycoprotein hormones has not been clearly elucidated. Using newly developed methods of enzymatic deglycosylation we have observed significantly decreased bioactivity of deglycosylated TSH, both in terms of potency and  $V_{max}$ . However, the effects vary in different assays, and are less than those previously reported for chemical deglycosylation. The mechanism for these effects appears to be related to a change in hormone conformation rather than a direct effect of carbohydrate with the receptor. Certain patients with thyrotropic tumors show serum TSH with enhanced bioactivity, probably resulting from altered glycosylation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 55002-09 MCNE
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. This must fit on one line between the borders.) <b>Molecular Biology of Glycoprotein Hormones</b>		
PRINCIPLE INVESTIGATOR (List other professional personnel below the Principle Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	B.D. Weintraub	Chief
		MCNEB, NIDDK
Others:	F.E. Wondisford	Senior Staff Fellow
	S. Usala	Medical Staff Fellow
	T. Taylor	Guest Researcher
	R.W. Lash	Medical Staff Fellow
	J. Steinfelder	Guest Researcher
	J. McClaskey	Senior Staff Fellow
		MCNEB, NIDDK
COOPERATING UNITS (if any)  None		
LAB/BRANCH Molecular, Cellular and Nutritional Endocrinology Branch		
SECTION Molecular Regulation and Neuroendocrinology Section		
INSTITUTE AND LOCATION NIDDK NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.3	5.3	0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  <p>We have localized DNA sequences in the human TSH-beta gene which mediate transcriptional regulation by thyroid hormone, thyrotropin-releasing hormone (TRH) and cyclic AMP. An element responsible for thyroid hormone inhibition of gene transcription is present in the downstream region of the first exon. In contrast, the elements mediating TRH and cAMP effects are present in the 5' flanking region. Site-directed mutagenesis of the coding region of the human TSH-beta gene shows that both the carbohydrate as well as specific amino acids in the CAGYC region are important for subunit combination and protection from intracellular degradation.</p> <p>The onset of thyrotropin-releasing hormone (TRH) response to thyroid hormone feedback corresponds to the time when TRH begins to regulate TSH secretion. The increase in fetal plasma TSH observed in late gestation is independent of hypothalamic TRH regulation.</p> <p>We have shown that thyroid hormone resistance is linked to the c-erbA-beta receptor gene in three families. In one family the disease appears to result from a single base change in the hormone binding domain of the receptor. The molecular basis for the disease appears different in two other families and is currently being elucidated.</p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE		PROJECT NUMBER
<b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		Z01 DK 55006-16 MCNEB
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. This must fit on one line between the borders.) <b>Insulin-like Growth Factors</b>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principle Investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.:	M.M. Rechler	Chief, GD Section MCNEB, NIDDK
Others:	A.L. Brown	Staff Fellow MCNEB, NIDDK
	D.E. Graham	Microbiologist MCNEB, NIDDK
	C.C. Orłowski	Staff Fellow MCNEB, NIDDK
	Y.W.-H. Yang	Staff Fellow MCNEB, NIDDK
	J.A. Romanus	Biologist MCNEB, NIDDK
	L. Tseng	Chemist MCNEB, NIDDK
	G.T. Ooi	Special Volunteer MCNEB, NIDDK
COOPERATING UNITS (if any) MB NCI (S.P. Nissley, W. Kiess, M.M. Sklar, M.C. Gelato); PB NCI (O.M. El-Badry, M.A. Israel); Univ. of Naples, Italy (C.B. Bruni, L. Chiariotti, R. Frunzio, C. Bucci); American Red Cross (W.H. Burgess, T. Mehlman)		
LAB/BRANCH Molecular, Cellular and Nutritional Endocrinology Branch		
SECTION Growth and Development Section		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS: 8	PROFESSIONAL: 6	OTHER: 2
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input checked="" type="checkbox"/> (b) Human tissues <input type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>We have continued our studies of the insulin-like growth factors (IGFs), their receptors and binding proteins to understand the role of the IGFs in physiological and pathological growth. During the past year we have demonstrated that: (1) multiple rat IGF-II mRNAs (1.0 to 4.6 kb) arise from a single gene through the use of 2 promoters and 2 polyA addition sites; (2) the IGF-II gene is transcribed from both promoters in multiple non-neural tissues in fetal rats and in fetal and adult rat brain; (3) IGF-II mRNA is expressed at high levels by a human neuroblastoma cell line, and by human neuroblastoma and pheochromocytoma tumors; (4) IGF-II synthesized by this human neuroblastoma cell line stimulates autonomous growth via the IGF-I receptor; (5) IGF-II inhibits the binding of a lysosomal enzyme, <math>\beta</math>-galactosidase to the IGF-II/Mannose 6-phosphate receptor, and may modulate the distribution and function of lysosomal enzymes; (6) the IGF-II/Man 6-P receptor is present at high levels in fetal rat tissues and in serum from fetal rats and monkeys, and decreases with developmental age; (7) the nucleotide sequence of an IGF-binding protein, BP-3A, indicates that it is one of a family of related proteins; (8) a single BP-3A mRNA of ~2 kb is expressed in multiple fetal rat tissues and adult rat brain; (9) a carboxyl-terminal fragment of BP-3A retains the ability to bind IGF-I and IGF-II, suggesting that it contains at least part of the IGF binding domain; (10) BP-3A is the predominant IGF binding protein in fetal rat serum; (11) the major 150 kDa IGF-binding protein complex in adult rat serum consists of a glycosylated IGF-binding subunit (BP-53) that associates with a nonbinding subunit in the presence and absence of IGFs; and (12) rat BP-53 contains two IGF-binding sites (on the same or different molecules) whose specificity is unaffected by enzymatic deglycosylation.</p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 55007-11 MCNE

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin-Cell Interaction

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	T. M. Weber	Staff Fellow	MCNEB, NIDDK
Others:	I. A. Simpson	Visiting Scientist	MCNEB, NIDDK
	S. W. Cushman	Chief, EDMNS	MCNEB, NIDDK

COOPERATING UNITS (if any) Department of Medicine, University of Bari, Bari, Italy (S. DiPaolo); Institute of Pharmacology and Toxicology, University of Göttingen, Göttingen, Germany (H. G. Joost); Fermentation Research Laboratories, Sankyo Company, Tokyo, Japan (M. Kuroda).

LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

SECTION

Experimental Diabetes, Metabolism and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

0.3

PROFESSIONAL:

0.3

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

The phosphorylation state and tyrosine kinase activity of insulin receptors in subfractions of insulin-treated rat adipose cells have been studied. The results suggest that insulin receptors retain their kinase activity on internalization, indicating that the receptor kinase may possibly mediate insulin's effects while inside the cell. However, if the internalized receptor kinase mediates insulin's effect on glucose transport, only a portion of its maximum activity appears to be necessary for full transport stimulation (<20%). Further, the difference in kinase activity among subfractions suggests that the receptor kinase in the low-density microsomes may be in the process of deactivation. The effects of isoproterenol and insulin on the subcellular distribution and phosphorylation state of insulin receptors have been investigated in rat adipose cells. The results suggest that isoproterenol augments insulin's effect on receptor internalization, but reverses its stimulatory effect on receptor phosphorylation state and tyrosine kinase activity in plasma membranes; the latter effects may account for the decreased sensitivity of the glucose transport response to insulin.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 55008-11 MCNE

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin's Regulation of Glucose Transport

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S. W. Cushman Chief, EDMNS MCNEB, NIDDK  
 Others: I. A. Simpson, Visiting Scientist; T. M. Weber, Staff Fellow; M. J. Zarnowski, Biologist; D. R. Yver, Chemist; A. D. Habberfield, Visiting Fellow; T. L. Jones, Medical Staff Fellow; J. Saltis, Visiting Fellow, S. Krief, Special Volunteer, K. Chundu, Special Volunteer; T. Davies, Special Volunteer and O. Gonzalez-Mulero, Medical Staff Fellow; all from MCNEB/NIDDK

C. Londos, Chief, MRS, and J. J. Egan, Staff Fellow, both from LCDB/NIDDK.

## COOPERATING UNITS (if any)

Institute of Pharmacology and Toxicology, University of Göttingen, Göttingen, Germany (H. G. Joost); INSERM Unit 177, Paris, France (M. Lavau, M. Guerre-Millo).

## LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

## SECTION

Experimental Diabetes, Metabolism and Nutrition Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

7.2

## PROFESSIONAL:

7.2

## OTHER:

0.0

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Insulin's rapid action to increase glucose transport is believed to occur primarily through the translocation of glucose transporters from an intracellular pool to the plasma membrane. To better understand the mechanism involved, we studied the role of protein synthesis in glucose transporter translocation by using the protein synthesis inhibitor, cycloheximide. The results suggest that insulin's stimulation of glucose transport and translocation of glucose transporters can occur without acute protein synthesis. The possible role of protein kinase C in the regulation of glucose transport in the rat adipose cell has been examined. While the results do not clarify the relationship between protein kinase C and the mechanism of insulin action, they do suggest that 1) protein kinase C activation induces the translocation of glucose transporters without a corresponding increase in glucose transport activity and 2) insulin and PMA appear to stimulate glucose transport in rat adipose cells through distinct but interactive mechanisms. In another separate study, the data suggest that 1) glucose transporters redistributed in response to PMA or vasopressin exhibit different intrinsic activities compared to glucose transporters from insulin-stimulated cells and 2) the phosphorylation state of glucose transporters may regulate their activity. The question of a long-term regulatory role of insulin on adipocyte glucose transporter content has been addressed using the differentiating or fully mature 3T3-F442A adipocytes. The results suggest that insulin plays a long-term regulatory role on cultured adipocyte glucose transporter content through a selective effect on the erythrocyte/HepG2/brain-type glucose transporter. A procedure for purification of the 45 kDa transport protein from rat brain has been developed. An  $\approx 5,000$ -fold purification of the rat brain glucose transporter has been achieved with a yield of 25%.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Alterations in Insulin's Action in Insulin-Dependent Diabetes Mellitus

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S. W. Cushman

Chief, EDMNS

MCNEB, NIDDK

## COOPERATING UNITS (if any)

Diabetes Unit, Beth Israel Hospital, Boston, MA (B. B. Kahn, J. S. Flier); Whitehead Institute for Biomedical Research, Cambridge, MA (M. J. Charron, H. F. Lodish); University of Texas, San Antonio, TX (L. Rossetti, R. A. DeFronzo).

## LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

## SECTION

Experimental Diabetes, Metabolism and Nutrition Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.7

## PROFESSIONAL:

0.7

## OTHER:

0.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Diabetes and its treatment with insulin in the rat result in dramatic changes in insulin-stimulated glucose transport activity and glucose transporter number in adipose cells. In addition, at least two genetically distinct glucose transporters coexist in adipose cells, one cloned from human hepatoma cells and rat brain (Hep G2/brain) and another from rat skeletal muscle, heart and adipose cells (adipose cell/muscle). Here we demonstrate differential regulation of these two glucose transporters in adipose cells of diabetic and insulin-treated diabetic rats and compare changes in the expression of each glucose transporter with marked alterations in insulin-stimulated glucose transport activity. The results suggest that 1) altered expression of the adipose cell/muscle glucose transporter forms the molecular basis for the dysregulated glucose transport response to insulin characteristic of diabetes, 2) the expression of two types of glucose transporters in rat adipose cells is regulated independently, and 3) alterations in mRNA levels are only part of the mechanism for in vivo regulation of the expression of either glucose transporter. Evidence has recently accumulated for a direct role of glucose, independent of insulin, in the regulation of cellular glucose transport. Moreover, we have demonstrated the reversal of in vivo insulin resistance in diabetic rats by normalization of hyperglycemia without any change in plasma insulin concentration. In the present study, the effect of correction of hyperglycemia on insulin's stimulatory action on glucose transport activity in adipose cells from diabetic rats has been examined. The data show that normalization of the plasma glucose concentration in the absence of insulin therapy in diabetic rats restores, or may even enhance, the in vitro adipose cell glucose transport response to insulin while normalizing in vivo insulin-mediated glucose disposal and suggest that the plasma glucose concentration is an important regulator of glucose transport activity in adipose cells, independent of the plasma insulin concentration.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

201 DK 55012-07 MCNE

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Insulin's Regulation of Hormone Binding

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S. W. Cushman Chief, EDMNS MCNEB, NIDDK

Others: I. A. Simpson Visiting Scientist MCNEB, NIDDK

COOPERATING UNITS (if any)

Research Laboratories, A. H. Robins Company, Richmond, VA (K. C. Appell).

LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

SECTION

Experimental Diabetes, Metabolism and Nutrition Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

0.1

PROFESSIONAL:

0.1

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

A comparison of insulin's effects on glucose transport and cell surface IGF-II receptors has been undertaken in rat adipose cells using 3-O-methylglucose transport as a measure of glucose transport activity and Scatchard analysis of IGF-II binding in the presence of KCN to determine cell surface IGF-II receptor number. The results demonstrate that while the characteristics of the stimulatory action of insulin on glucose transport activity and cell surface IGF-II receptor number are qualitatively similar, quantitative differences are clearly demonstrable which suggest that the subcellular cycling of these two integral membrane proteins occurs by distinct processes. The effects of adenosine, isoproterenol, and glucose have now been examined on both steady state insulin responsiveness and sensitivity in this cell type prepared in the presence of saturating adenosine (200 nM). The results show that the stimulatory effect of insulin on IGF-II binding to rat adipose cells is modulated not only by counterregulatory hormones, but also by glucose, a major substrate of insulin action.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Counterregulation of Insulin's Action by Catecholamines

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	I. A. Simpson	Visiting Scientist	MCNEB, NIDDK
Others:	J. Saltis	Visiting Fellow	MCNEB, NIDDK
	A. D. Habberfield	Visiting Fellow	MCNEB, NIDDK
	H. Nishimura	Visiting Fellow	MCNEB, NIDDK
	S. W. Cushman	Chief, EDMNS	MCNEB, NIDDK
	M. J. Zarnowski	Biologist	MCNEB, NIDDK
	C. Londos	Chief, MRS	LCDB, NIDDK

## COOPERATING UNITS (if any)

Department of Medicine, University of Gothenburg, Gothenburg, Sweden (P. N. Lönnroth, U. Smith); Research Laboratories, A. H. Robins Company, Richmond, VA (K. C. Appell).

## LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

## SECTION

Experimental Diabetes, Metabolism and Nutrition Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.2

## PROFESSIONAL:

1.2

## OTHER:

0.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Insulin shifts the steady state subcellular distribution of IGF-II receptors from a large intracellular pool to the plasma membrane in the rat adipose cell. In the present study, the counterregulatory effects of adrenergic stimulation, adenosine deaminase (ADA), and cAMP on this process have been studied. The results suggest that  $\beta$ -adrenergic stimulation, through a cAMP-dependent mechanism, markedly alters the insulin-stimulated redistribution of IGF-II receptors. This effect is additional to the potent antagonistic action of cAMP on insulin's signaling mechanism. We have also examined the modulation of protein kinase C-mediated stimulation of glucose transport activity in the rat adipose cell by 1) ligands for receptors that mediate stimulation ( $R_s$ ; lipolytic) or inhibition ( $R_i$ ; antilipolytic) of adenylate cyclase and 2) pertussis and cholera toxins which regulate the inhibitory ( $G_i$ ) and stimulatory ( $G_s$ ) and stimulatory ( $G_s$ ) guanyl nucleotide-binding proteins of adenylate cyclase, respectively. The results suggest that 1) G-proteins modulate protein kinase C-mediated stimulation of glucose transport activity by altering the intrinsic activity of glucose transporters residing in the plasma membrane and 2) a functional  $G_i$ -protein is required for stimulation of glucose transport activity by phorbol esters and vasopressin.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 55014-06 MCNE

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Alterations in Insulin's Action with Fasting/Refeeding

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: S. W. Cushman Chief, EDMNS MCNEB, NIDDK

Others: I. A. Simpson Visiting Scientist MCNEB, NIDDK

## COOPERATING UNITS (if any)

Diabetes Unit, Beth Israel Hospital, Boston, MA (B. B. Kahn, J. S. Flier); Research Laboratories, A. H. Robins Company, Richmond, VA (K. C. Appell).

## LAB/BRANCH

Molecular, Cellular and Nutritional Endocrinology Branch

## SECTION

Experimental Diabetes, Metabolism and Nutrition Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.3

## PROFESSIONAL:

0.3

## OTHER:

0.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

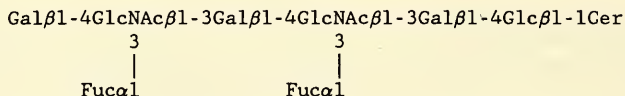
## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

The effects of fasting and refeeding on the glucose transport response to insulin in isolated rat adipose cells have been examined. The results suggest that the insulin resistant glucose transport in isolated adipose cells from fasted rats can be explained by a decreased translocation of glucose transporters to the plasma membrane due to a depleted intracellular pool. In contrast, the insulin hyperresponsive glucose transport observed with refeeding appears to result from 1) a restored translocation of glucose transporters to the plasma membrane from an intracellular pool replenished through an increase in intracellular protein and 2) enhanced plasma membrane glucose transporter intrinsic activity. The human Hep G2 glucose transporter cDNA clone has been used to examine the molecular basis for these alterations. The data suggest that the abundance of mRNAs for multiple adipose cell genes is affected by fasting and refeeding. In particular, this is the first demonstration in an insulin-sensitive tissue that glucose transporter number, and hence a major factor in the glucose transport response to insulin, may be controlled, at least in part, by alterations in mRNA abundance. Insulin increases glucose transport activity and IGF-II binding in rat adipose cells by eliciting the redistribution of glucose transporters and IGF-II receptors from large intracellular pools to the plasma membrane. We now have measured cell surface IGF-II binding in intact cells from 2-day fasted and 2-day fasted/6-day refed rats and assessed IGF-II receptor number in subcellular membrane fractions by immunoblotting. The results suggest that fasting differentially regulates the number and distribution of IGF-II receptors and glucose transporters in adipose cells. This finding suggests distinct intracellular trafficking pathways for these proteins. Nutritional regulation of the IGF-II receptor may serve as a tool to explore the physiological role of IGF-II.

ANNUAL REPORT OF THE LABORATORY OF STRUCTURAL BIOLOGY  
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

I. BIOLOGY OF COMPLEX CARBOHYDRATES

Immunocytochemical staining of cells in sputum by rat monoclonal antibody 624H12 detects lung cancer two years prior to its detection by conventional diagnostic techniques. The antigen recognized by antibody 624H12 is a sugar sequence in the glycosphingolipid difucosylneolactonorhexaosylceramide ( $V^3\text{FucIII}^3\text{FucnLc6Cer}$ ) whose structure is:



Both fucosyl residues are required for high affinity binding by the antibody. The antigen was expressed in 35 out of 45 specimens of cancer tissue from patients with early stage non small cell lung cancer. There was no correlation between antigen expression and patient survival.

....Drs. V. Ginsburg, N.H. Guo, L. Zhang, M. Kyogashima, H. Krivan and G. Holt

II. METABOLISM AND ROLE OF POLYSACCHARIDE SULFATES

Fucoidan, a fucose containing sulfated carbohydrate polymer, inhibits a wide range of physiological processes which involve cell interactions such as sperm-egg fertilization, tumor growth and the binding of lymphocytes to the endothelial cells of post-capillary venules. The preparation of polysulfated methyl fucosides of known structure will help elucidate the mechanism of these cell surface phenomena.

Many polyanions bind to normal and sickle hemoglobin and affect its solubility. Highly sulfated trehalose has been shown to increase the solubility of hemoglobin-S. The synthesis of sulfated anions of appropriate size, which may have significant anti-sickling properties, is being pursued.

....Dr. I. Leder

### III. EXPRESSION AND FUNCTION OF BACTERIAL CELL SURFACE COMPONENTS IN PATHOGENESIS

The bacterial cell surface functions both in the physiology of the cell but also in the interaction of the cell with its environment. For human pathogens, these include adhesion, host invasion and subsequent evasion of host defense mechanisms such as killing by serum, phagocytosis, and interactions with specific antibodies. The amount and specific structure of individual bacterial cell surface components determine these properties. For example, two structures found on the surface of Gram-negative bacteria are lipopolysaccharide (LPS) and capsular polysaccharide. LPS that contain long side chains of O-antigen polysaccharide are found on bacteria that resist bacteriocidal effects of serum. Similarly, the capsular polysaccharide may serve to protect the bacterial cell against the killing effects of serum. This protection is due to the ability of these polysaccharides to interfere with the rate and/or extent of deposition of the complement component C3b on the bacterial cell surface. This deposition is a prerequisite for subsequent events leading to the death of the bacterial cell. Salmonellae typhi is the agent responsible for typhoid fever in man and the surface of these cells is coated with two polysaccharides, LPS and the capsular polysaccharide called the Vi-antigen. We have examined the relative roles of the Vi-antigen and the O-antigen polysaccharide side chains of LPS in resistance of iogenic Vi<sup>+</sup> strains of Salmonellae typhi to determine the relative contributions of surface structures in the protection of the cell against serum killing and phagocytosis. We found the Vi-antigen had no influence on resistance to serum killing but decreases the relative rate of phagocytosis of Vi<sup>+</sup> cells. We suggest Vi-antigen may interfere with the recognition of complement on the bacterial cell surface by phagocyte receptors. We also examined the effect of aspirin on antibiotic sensitivity of bacteria, and find that cells grown in the presence of 1-5 mM aspirin (or tylenol) become resistant to levels of antibiotics commonly achieved during therapy. This resistance is due to inhibition of expression of cell surface protein, the OmpF porin, that functions the uptake of the antibiotic. This suggests that administration of aspirin or tylenol with commonly prescribed antibiotics be avoided.

..... Drs. J. Foulds, V. Jimenez

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 57000-24 LSB

## PERIOD COVERED

October 1, 1988 through September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biology of Complex Carbohydrates

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Victor Ginsburg, Ph.D.

Chief, LSB

NIDDK

Others: Neng Hua Guo, M.D.

Visiting Fellow

LSB

NIDDK

Lijuan Zhang

Visiting Fellow

LSB

NIDDK

Mamoru Kyogashima, Ph.D., M.D.

Visiting Fellow

LSB

NIDDK

Howard Krivan, Ph.D.

Staff Fellow

LSB

NIDDK

Gordon Holt, Ph.D.

Staff Fellow

LSB

NIDDK

## COOPERATING UNITS (if any)

James L. Mulshine, NCI

## LAB/BRANCH

Laboratory of Structural Biology

## SECTION

Section on Biochemistry

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

7

## PROFESSIONAL:

6

## OTHER:

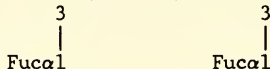
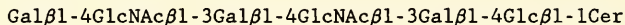
1

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Immunocytochemical staining of cells in sputum by rat monoclonal antibody 624H12 detects lung cancer two years prior to its detection by conventional diagnostic techniques. The antigen recognized by antibody 624H12 is a sugar sequence in the glycosphingolipid difucosylneolactonorhexaosylceramide ( $V^3$ FucIII $^3$ FucnLc6Cer) whose structure is:



Both fucosyl residues are required for high affinity binding by the antibody. The antigen was expressed in 35 out of 45 specimens of cancer tissue from patients with early stage non small cell lung cancer. There was no correlation between antigen expression and patient survival.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 57001-12 LSB

PERIOD COVERED

October 1, 1988 through September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Metabolism and Role of Polysaccharide Sulfates

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Irwin G. Leder

Research Chemist

LSB NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Structural Biology

SECTION

Section on Biochemistry

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1

PROFESSIONAL:

1

OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects

☐ (b) Human tissues

☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Fucoidan, a fucose containing sulfated carbohydrate polymer, inhibits a wide range of physiological processes which involve cell interactions such as sperm-egg fertilization, tumor growth and the binding of lymphocytes to the endothelial cells of post-capillary venules. The preparation of polysulfated methyl fucosides of known structure will help elucidate the mechanism of these cell surface phenomena.

Many polyanions bind to normal and sickle hemoglobin and affect its solubility. Highly sulfated trehalose has been shown to increase the solubility of hemoglobin-S. The synthesis of sulfated anions of appropriate size, which may have significant anti-sickling properties, is being pursued.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 57002-15 LSB
PERIOD COVERED October 1, 1988 through September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <b>Expression and Function of Bacterial Cell Surface Components in Pathogenesis</b>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI:	John Foulds	Research Biochemist      LSB    NIDDK
Others:	Victor Jimenez, M.D., M.Sc.	Staff Fellow      LSB    NIDDK
COOPERATING UNITS (if any) Judeh Rosner, LMB, NIDDK		
LAB/BRANCH	Laboratory of Structural Biology	
SECTION	Section on Biochemistry	
INSTITUTE AND LOCATION	NIDDK, NIH, Bethesda, Maryland 20892	
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard units of work. Do not exceed the space provided.) <p>           The bacterial cell surface functions both in the physiology of the cell but also in the interaction of the cell with its environment. For human pathogens, these include adhesion, host invasion and subsequent evasion of host defense mechanisms such as killing by serum, phagocytosis, and interactions with specific antibodies. The amount and specific structure of individual bacterial cell surface components determine these properties. For example, two structures found on the surface of Gram-negative bacteria are lipopolysaccharide (LPS) and capsular polysaccharide. LPS that contain long side chains of O-antigen polysaccharide are found on bacteria that resist bacteriocidal effects of serum. Similarly, the capsular polysaccharide may serve to protect the bacterial cell against the killing effects of serum. This protection is due to the ability of these polysaccharides to interfere with the rate and/or extent of deposition of the complement component C3b on the bacterial cell surface. This deposition is a prerequisite for subsequent events leading to the death of the bacterial cell. <u>Salmonellae typhi</u> is the agent responsible for typhoid fever in man and the surface of these cells is coated with two polysaccharides, LPS and the capsular polysaccharide called the Vi-antigen. We have examined the relative roles of the Vi-antigen and the O-antigen polysaccharide side chains of LPS in resistance of iogenic Vi<sup>+</sup>/<sub>-</sub> strains of <u>Salmonellae typhi</u> to determine the relative contributions of surface structures in the protection of the cell against serum killing and phagocytosis. We found the Vi-antigen had no influence on resistance to serum killing but decreases the relative rate of phagocytosis of Vi<sup>+</sup> cells. We suggest Vi-antigen may interfere with the recognition of complement on the bacterial cell surface by phagocyte receptors. We also examined the effect of aspirin on antibiotic sensitivity of bacteria, and find that cells grown in the presence of 1-5 mM aspirin (or tylenol) become resistant to levels of antibiotics commonly achieved during therapy. This resistance is due to inhibition of expression of cell surface protein, the OmpF porin, that functions the uptake of the antibiotic. This suggests that administration of aspirin or tylenol with commonly prescribed antibiotics be avoided.         </p>		

ANNUAL REPORT OF THE  
MOLECULAR, CELLULAR, AND NUTRITIONAL ENDOCRINOLOGY BRANCH  
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

The IMCB comprises several groups. One group, led by Dr. Carter, studies gene regulation in mammalian cell systems and is particularly interested in developing efficient vector systems for delivery of genes into cells. The second group, led by Dr. Oka, is generally interested in the endocrine control of differentiation of the mouse mammary gland and has focused on physiological effects of EGF and the molecular biology of various genes which are important in this process. A third project led by Dr. Tietze, is aimed at understanding the molecular basis of several human genetic defects which result in lysosomal storage diseases. This project is conducted in collaboration with workers in NICHD.

Function of DNA Virus Genomes in Animal Cells

The group led by B. Carter has continued to employ DNA viruses as molecular probes to study genome expression in human cells. We are studying intensively the structure and function of adeno-associated virus (AAV) since this virus has only a small genome. AAV has also been developed as a eukaryotic expression vector. AAV normally grows in cells only in the presence of a helper virus (either adenovirus or herpesvirus). In the absence of any helper the AAV genome integrates into the cell chromosome. Thus, the AAV vector is useful as a transducing virus for high frequency integration of genes in mammalian cell chromosomes to yield stable expression. This vector also may be useful for therapy. A patent for this vector system was awarded in January, 1989. We are now analyzing intensively the control of gene regulation in AAV vectors in order to maximize the expression of foreign genes introduced into mammalian cells using this vector. We have discovered a complex system of gene regulation mediated by products of the AAV rep gene which are required for replication of AAV DNA but also mediate transcriptional activation and also translational inhibition of some genes. We are using site-directed mutagenesis to resolve the functions of the rep gene. Coding of all these functions in a single gene appears to be unique in eukaryotic systems. We are also studying adenovirus since this is the helper virus for AAV multiplication. This helper relationship is being analyzed. Also, both AAV and adenovirus recombine with cellular DNA. In the case of adenovirus, this causes malignant transformation of the cell. AAV inhibits this transformation and also inhibits Ad12 oncogenesis in newborn animals. The mechanism of this inhibition of tumor induction by AAV is being studied at the molecular level in both cell culture and in animal experiments. We are also studying mutations in mouse 3T3 cells which render the cells resistant to malignant transformation by a single oncogene (ras) but allow malignant transformation by two oncogenes (ras, myc) acting in concert.

We are also studying regulation of HIV by AAV. Current work suggests that production of vaccines or use of nucleotide analog therapies for AIDS may be limited and difficult. Thus other therapeutic approaches are urgently required. We are attempting to develop a novel approach by using the negative regulatory property of a trans-acting gene of the human parvovirus, AAV, to

inhibit the function of the trans-acting gene tat, of HIV. A functional tat gene is required for HIV growth so inhibition of tat presents a potentially useful approach to an antiviral therapy.

### Hormonal Regulation of Cell Growth and Differentiation

Epidermal growth factor (EGF) is produced in large amounts by the mouse submandibular gland. It is also present in such biological fluids as plasma, milk, urine and saliva. EGF is a potent mitogen for a wide variety of cells in culture but its function in the body needs to be elucidated. Dr. Oka's studies have demonstrated that EGF plays a key role in the development of the mammary gland during pregnancy and mammary tumorigenesis in female mice; in males it serves a role in spermatogenesis by stimulating the meiosis of spermatocytes. Studies have been continued to elucidate the physiological role of EGF by employing a variety of experimental approaches, including radioimmunoassay of EGF in tissues and biological fluids, EGF receptor assay and bioassay of EGF in cell culture. In addition, a useful means of causing EGF deficiency in mice by removal of the submandibular gland and/or administration of anti-EGF antiserum has been established. These procedures, combined with EGF replacement therapy have provided valuable information concerning the function of EGF in the body. These studies have shown that the concentration of EGF in the submandibular gland and plasma of female mice increases significantly during pregnancy. Attenuation of the rise in EGF by sialoadenectomy and anti-EGF treatment resulted in increased rate of spontaneous abortion, suggesting that EGF is necessary for the normal course of pregnancy. In addition, EGF has been shown to have a physiological role in maintaining the normal structure of the epidermis. Additional studies also have revealed that milk contains a high concentration of EGF which serves a physiological function by promoting neonatal eyelid opening.

### Lysosomal Transport and Storage Disease

This work is being conducted by Dr. Frank Tietze. Degradation of cellular biopolymers such as proteins and polysaccharides takes place chiefly within the lysosome. The end-products of this degradation, viz., amino acids and monosaccharides, are presumed to exit the lysosome to the cytoplasm, where further metabolism or expulsion to the external medium occurs. To study the process of lysosomal transport, methods were developed to load lysosomes of various cells with amino acids (e.g., cystine, tyrosine) or with a specific monosaccharide (viz., sialic acid) and to measure their rates of egress from the organelle. Studies of cystine egress from lysosomes of human polymorphonuclear leukocytes and of tyrosine from cultured rat thyroid cell lysosomes have revealed these processes to be carrier-mediated and stereospecific. The further demonstration that no egress of cystine could be detected from similarly loaded lysosomes from patients with the inherited disorder cytinosis indicated that this storage disease is due to a congenital defect of a specific lysosomal carrier. Similar studies on the egress of sialic acid from fibroblast lysosomes have suggested strongly that impaired lysosomal transport underlies another lysosomal storage disorder, free sialic acid storage disease. In addition to a carrier system specific for the lysosomal transport of tyrosine, preliminary evidence has indicated that lysosomes from cultured rat thyroid cells also possess a carrier for moniodotyrosine an end-product of the lysosomal catabolism of thyroglobulin.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 57501-13 LMCB
PERIOD COVERED October 1, 1988 through September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Function of DNA Virus Genomes in Animal Cells		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal investigator.) (Name, title, laboratory, and institute affiliation)		
P.I.:	Barrie J. Carter	Chief, LMCB LMCB:NIDDK
Other:	James Trempe	Senior Staff Fellow LMCB:NIDDK
	Nor Chejanovsky	Visiting Associate LMCB:NIDDK
	Irving Miller	Biologist LMCB:NIDDK
	Beth Antoni	IRTA Fellow LMCB:NIDDK
	Roland Owens	IRTA Fellow LMCB:NIDDK
	John Smuda	IRTA Fellow LMCB:NIDDK
	Terry Flotte	NIH-Johns Hopkins CF Fellow LMCB:NIDDK
COOPERATING UNITS (if any)		
M.G. Smith, Univ. Otago, New Zealand; J. Tal, Beersheba, Israel; A.R. Rabson (NIAID), D.F. Klessig, Rutgers		
LAB/BRANCH Laboratory of Molecular and Cellular Biology		
SECTION		
INSTITUTE AND LOCATION NIDDK:NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7	7	
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither		
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)		
<p>           We are employing <u>DNA viruses</u> as <u>molecular probes</u> to study genome expression in <u>human cells</u>. We are studying intensively the structure and function of a <u>human parvovirus</u>, <u>adeno-associated virus (AAV)</u> since this virus has only a small genome. AAV has been developed as a <u>eukaryotic expression vector</u>. AAV normally grows in cells only in the presence of a helper virus (either <u>adenovirus</u> or <u>herpesvirus</u>). In the absence of any helper, the AAV genome <u>integrates</u> into the <u>cell chromosome</u>. Thus, the AAV vector is useful as a <u>transducing virus</u> for high frequency integration of genes into <u>mammalian cell chromosomes</u> to yield stable expression. This vector also may be useful for <u>gene therapy</u>. We are now analyzing intensely the control of <u>gene regulation</u> in AAV vectors in order to maximize the <u>expression of foreign genes</u> introduced into <u>mammalian cells</u> using this vector. We have discovered a complex system of <u>gene regulation</u> mediated by products of the AAV rep gene which are required for <u>replication of AAV DNA</u> but also mediate <u>transcriptional activation</u> and <u>translational inhibition</u> of some genes. <u>Site-specific mutagenesis</u> and being used to resolve these functions. Coding of all these functions in a single gene is unique in eukaryotic systems. Adenovirus is the helper for AAV. This relationship is being analyzed. Both AAV and adenovirus recombine with cellular DNA. In the case of adenovirus this causes <u>malignant transformation</u> of the cell. AAV inhibits this transformation and also inhibits <u>Ad12 oncogenesis</u> in newborn animals. Thus, AAV inhibits <u>tumor induction</u>. The mechanism of this inhibition of tumor induction is being studied at the molecular level in cell culture. We also are analyzing interactions of AAV with HIV as a potential approach to a <u>novel therapy</u> for <u>AIDS</u>.         </p>		

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 57502-16 LMCB

## PERIOD COVERED

October 1, 1988 through September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Hormonal Regulation of Cell Growth and Differentiation

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. Takami Oka Senior Investigator LMCB:NIDDK

Other: Soji Kasayama	Visiting Fellow	LMCB:NIDDK
Chang-Soo Lee	Visiting Fellow	LMCB:NIDDK
Yoshito Ohba	Guest Worker	LMCB:NIDDK
John W. Perry	Biologist (Technician)	LMCB:NIDDK
Katsuya Wada	Guest Worker/Visiting Fellow	LMCB:NIDDK
Masami Yoshimura	Visiting Associate	LMCB:NIDDK

## COOPERATING UNITS (if any)

Dr. Kishio Furuya, National Institute of Physiological Sciences, Japan

## LAB/BRANCH

Laboratory of Molecular and Cellular Biology

## SECTION

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

6.5

## PROFESSIONAL:

5.5

## OTHER:

1.0

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Epidermal growth factor (EGF) is a single-chain polypeptide which exerts a potent action on the growth and differentiation of various types of mammalian cells. EGF is produced in large amounts in the mouse submandibular gland, while small amounts of EGF are also found in the kidney and other tissues. EGF is also present in the plasma and other biological fluids such as tears, milk and saliva. Our previous studies have indicated that EGF plays an important function in maintenance of normal pregnancy, spermatogenesis and epidermal structure, promoting neonatal eyelid opening and corneal wound healing as well as mammary tumorigenesis. Thus, the possibility exists that abnormal production of EGF and/or alterations in EGF receptor contributes to the pathology of disease states. Accordingly, we have examined the regulation of EGF gene expression and its receptor level in normal and a certain disease state, i.e. diabetes mellitus. Our studies have shown that EGF gene expression in the submandibular gland is dependent on androgen, and thyroid hormones, and that the rise in plasma EGF levels is under the influence of androgen. The expression of hepatic EGF receptor gene in genetically diabetic mice is reduced more than 70% when compared to that in normal controls. In addition, the production of NGF in the submandibular gland of diabetic mice is greatly depressed. The defect in EGF receptor gene expression and NGF production in diabetic mice can be corrected by insulin treatment, suggesting that insulin is important for the regulation of the growth factor and its receptor gene expression. These results suggest a functional connection between insulin and growth factor which may be disrupted in diabetes mellitus, leading to manifestation of pathological complications.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 57503-16 LMCB
PERIOD COVERED October 1, 1988 through September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Studies on the Metabolic Defect in Sialuria		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <div style="display: flex; justify-content: space-between; margin-top: 10px;"> <span>P.I. Frank Tietze</span> <span>Research Chemist</span> <span>LMCB:NIDDK</span> </div>		
COOPERATING UNITS (if any) William A. Gahl, Human Genetics Branch, NICHD Gilbert Ashwell, Laboratory of Biochemistry and Metabolism, NIDDK		
LAB/BRANCH Laboratory of Molecular and Cellular Biology		
SECTION		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 1.0	OTHER: -
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div> <input type="checkbox"/> (a) Human subjects  <input type="checkbox"/> (a1) Minors  <input type="checkbox"/> (a2) Interviews         </div> <div> <input checked="" type="checkbox"/> (b) Human tissues         </div> <div> <input type="checkbox"/> (c) Neither         </div> </div>		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Several inborn errors of metabolism involving <u>sialic acid</u> (N-acetyl-neuraminic acid; NANA) are known. In one category of disorder, i.e., Salla disease and infantile sialic acid storage disease (ISSD), there is a massive accumulation of free NANA within the <u>lysosomal</u> compartment of the patient's cells due to the inability of the monosaccharide to traverse the lysosomal membrane. Another category of storage disorder involving sialic acid is exemplified by sialuria of the "French type", in which the intracellular storage of sialic acid is accompanied by massive urinary excretion of the sugar. We studied cultured fibroblasts from two patients with sialuria, with the following results: (1) Unlike the intralysosomal storage in cells from Salla and ISSD patients, the storage of free NANA in sialuria fibroblasts is confined principally to the high speed (cytosolic) supernatant fraction. (2) Changes in the intracellular levels of free NANA in cells exposed to the sialic acid precursors glucosamine and N-acetyl-D-mannosamine are consistent with a defect in the feedback control of the rate-limiting enzyme in sialic acid biosynthesis, <u>UDP-N-acetylglucosamine-2-epimerase</u>. (3) Direct enzyme assay of the epimerase in extracts of the two mutant sialuria cell lines showed a strikingly diminished sensitivity to inhibition by CMP-NANA, the activated end product of sialic acid biosynthesis and the presumed natural feedback regulator of intracellular free sialic acid levels; this aberrant behavior was in marked contrast to the strong inhibition exhibited by the enzyme of normal cells in the presence of the same feedback inhibitor. It is concluded that the basic biochemical defect in sialuria of the "French type" is the loss of <u>feedback control</u> of UDP-GlcNAc epimerase by CMP-NANA, resulting in cellular overproduction of free sialic acid.</p>		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 DK 57504-02 LMCB
PERIOD COVERED October 1, 1988 through September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Regulation of HIV by AAV		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) P.I.: Barrie J. Carter Chief, LMCB LMCB:NIDDK  Other: Irving L. Miller Biologist LMCB:NIDDK Beth Antoni IRTA Fellow LMCB:NIDDK		
COOPERATING UNITS (If any) A.S. Rabson (LMM, NIAID)		
LAB/BRANCH Laboratory of Molecular and Cellular Biology		
SECTION		
INSTITUTE AND LOCATION NIDDK:NIH, Bethesda, Maryland 20892		
TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 1.0	OTHER: -
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>           The etiologic agent of AIDS is the <u>human immunodeficiency virus</u> (HIV) which differs from most other <u>human viral diseases</u> in exhibiting a very <u>prolonged latent period</u>, but ultimately being lethal due to a profound effect on the <u>immune system</u>. Several <u>trans-acting HIV genes</u> appear to be crucial to HIV growth and infection. Therefore we are studying the feasibility of a novel <u>anti-viral therapy</u> for HIV based on interference by another viral with the <u>trans-acting regulation</u> of HIV. The overall goal of this proposal is to analyze interactions between trans-acting regulatory genes of HIV and of a <u>human parvovirus, adeno-associated virus, AAV</u>. We are analyzing the AAV <u>rep</u> gene and its interaction with the HIV <u>tat</u> gene. Current work suggests that developing standard types of anti-viral therapy such as vaccines or nucleotide-analog drugs for HIV is difficult and other alternate possibilities for therapy must be investigated. One approach is to intervene in the <u>trans-regulation system</u> of HIV especially that mediated by the HIV <u>tat</u> gene. Thus a possible anti-viral therapy for HIV is to inhibit the production or the action of <u>tat</u>. A novel way to attempt this is to employ a <u>trans-acting gene</u> from another human virus. One such candidate is the <u>rep</u> gene of the human parvovirus adeno-associated virus (AAV). AAV does not cause any human disease and grows only in cells also infected with <u>adenovirus</u> or <u>herpes viruses</u>. AAV inhibits growth of the helper virus and may play an important role in limiting certain <u>human viral infections</u>. Also AAV can alter important regulatory controls in virus infected cells or in <u>tumor cells</u>. <u>Rep</u> is a novel type of <u>trans-acting regulatory gene</u> which exhibits negative, translational regulation of many genes in several cell types. We are analyzing the AAV <u>rep</u> gene and its interaction with the HIV <u>tat</u> gene. We are testing <u>rep</u> as a potential inhibitor of HIV infection or growth by interfering with trans-acting HIV genes.         </p>		

## ANNUAL REPORT OF THE LABORATORY OF ANALYTICAL CHEMISTRY

## NATIONAL INSTITUTE OF DIABETES, DIGESTIVE AND KIDNEY DISEASES

## SECTION ON INSTRUMENTATION

Basic research and service functions are performed by members of the Section. A major mission of the organization involves the instrumental and chemical analyses provided to NIDDK scientists of the, Laboratory of Bioorganic Chemistry, other NIH laboratories and to a limited extent to personnel of other government agencies. Instrumental analyses include: GC/MS spectrometry, HPLC/MS spectrometry, infrared spectrometry, nuclear magnetic resonance spectrometry, and element detection using a microwave plasma detection system. Assistance in interpretation of spectra is rendered on request. Samples of micro analysis are handled through external contracts. (D.F. Johnson, H.J.C. Yeh, N. Whittaker, W. White.

## APPLICATIONS OF NMR IN BIOCHEMICAL AND BIOLOGICAL SYSTEMS

NMR spectroscopy has been used to elucidate the structures of covalent adducts formed from DNA and a series of optically active bay-region epoxide derived from polycyclic aromatic hydrocarbons. We expect that this information will provide points of reference for further studies on the detailed structural (e.g. sequence, conformation) requirements for adduct formation between DNA and specific diol epoxides of known structure. (H.J.C. Yeh D.M. Jerina, J. M. Sayer, H. Yagi, L.K. Pannell, A. Chadha, And A.M. Cheh)

$^{19}\text{F}$  NMR spectroscopy has been used to study the interaction between the anaesthetic halothane and rat brain tissue in an attempt to understand the molecular mechanism of general anaesthetic action. It was found that halothane concentration in brain correlated with the inspired halothane concentration. We also found that halothane molecules are highly immobilized in a chemical environment characterized by a single short  $^{19}\text{F}$  spin-spin relaxation time in brain ( $T_2=4.2$  ms). These data tend to support the theory that general anaesthetics act by non specific perturbation of the nerve membranes rather than act by binding to unique, saturable sites on the neuronal membrane as reported earlier. (H.J.C. Yeh P. Skolnick, E. Moody)

## SECTION ON STEROID HORMONES

The objective of this project is to define the initial, intracellular events of glucocorticoid hormone action, and steroid hormone action in general. These events include steroid binding to the intracellular receptor molecule, activation of the receptor-steroid complex to a DNA-binding and nuclear-binding species, and binding of activated complexes to those nuclear acceptor sites involved in the regulation of transcription of specific genes. A combination of techniques have been used to examine the first two steps for glucocorticoid steroids. Studies with the affinity label dexamethasone mesylate (Dex-Mes) and the thiol-specific reagent methyl methanethiolsulfonate (MMTS) have revealed the

involvement of two thiol groups in steroid binding. Contrary to the current dogma, we found that reduced thiols are not absolutely required for binding to glucocorticoid receptors if the steric bulk of the oxidized thiols is small. We raised polyclonal antibodies against a defined epitope near the DNA-binding domain of the receptor. These antibodies recognized only activated complexes, thus providing direct evidence for a topological change in the receptor resulting from activation. Thiol reagents were also used to establish, for the first time, functional heterogeneity among activated complexes. Thus MMTS inhibited the DNA binding of one sub-class complexes while arsenite, which is specific for vicinal dithiols, blocked the DNA binding of the other sub-class. Furthermore, one sub-class of complexes undergoes a two-step activation process in which the association of a small molecular weight (700-3000 daltons) factor is required for DNA binding. Further studies using these techniques along with molecular biology should provide new information about steroid hormone control of gene transcription at a molecular level.

#### THE DEVELOPMENT OF METHODS AND MATERIALS FOR THE STUDY OF MEDICAL PROBLEMS

The objective of this project is to make contributions to the investigation and solution of basic biological and medical problems by the application of chemical, physical and biological methods. Lethality from cancer frequently results from metastases. Of tumor cells which enter the circulation less than one percent are successful in negotiating the steps of metastasis. This vulnerability may afford opportunities for selectively inhibiting the process. The purposes of this study are to increase our knowledge of the biology and chemistry of metastasis and to study the effect of selected biologicals and chemicals on the process. Such studies will also contribute to the investigation of other biological and medical problems.

The stable phenotypes of many malignant cells suggest that there is a genetic basis for cancer. However, the population of malignant cells in a tumor is heterogeneous and the cells vary in metastatic potencies. Epidemiological studies and recent studies with oncogenes suggest that carcinogenesis is a multistep process. The cancer phenotype in metastasis does not represent the initial change in growth control leading to tumorigenicity. If one or more additional genetic events are required for the metastatic phenotype, they may provide approaches to the prevention or treatment of metastasis.

NIH 3T3 cells, non-tumorigenic murine cells, have been transfected with a pBR322 plasmid bearing the src gene. Transformed cells bearing the src gene were injected into nude mice subcut and iv to test for tumorigenic, metastatic and lung-colonizing capabilities. NIH 3T3 cells were similarly transfected with constructs of the v-abl, c-mos, and v-mos oncogenes. Three cell lines developed with the src gene were tumorigenic but not metastatic and had poor lung colonizing potency. Three lines of transformed cells developed from v-mos transfected cells have also been tested in nude mice. Of cell lines derived from NIH 3T3 cells by transfections with src and v-mos oncogenes and tested in nude mice, all are tumorigenic, not metastatic, and weak in producing lung colonies. These cell lines appear to be good candidates for further transfections to determine whether greater metastatic and/or greater

lung-colonizing capabilities can be developed in this way. Positive results might afford new routes for the prevention and treatment of metastases. Other means for interfering with metastasis at the various steps of the process are also being sought using murine tumor cells, such as the Lewis lung carcinoma and PMT fibrosarcoma cells. (C.M. Foltz, L.A. Liotta, R. Muschel).

## SECTION ON BIOPHYSICAL HISTOLOGY

### A RHODAMINE FOR INTRACELLULAR INJECTION

Studies on neuronal structure in isogenic snails, on the synthesis of a new rhodamine dye, and on the possible use of this dye as an intracellular tracer have been interrupted. (W. W. Stewart and N. Feder).

### GENETICS OF NERVE CELL SHAPE

Studies are continuing on the genetics of *Biomphalaria glabrata*, a snail well suited to examining the genetic determinants of neuron structure and function. We have produced over a hundred highly inbred strains and have found about ten morphologic markers with a simple genetic basis. (W. W. Stewart and N. Feder).

### PROFESSIONAL PRACTICES OF BIOMEDICAL SCIENTISTS

Studies are continuing on the profession practices of scientists and on the accuracy of the scientific literature. The accuracy of an article in molecular biology is under investigation by ourselves and others. We have completed a manuscript on this subject and submitted it to three scientific journals. It has been under consideration by one journal for over a year, but has not yet been accepted for publication. We have also testified before two congressional committees on the subject of professional practices and the accuracy of the scientific literature. (W. W. Stewart and N. Feder).

## SECTION ON BIOMEDICAL CHEMISTRY

### NUCLEOSIDE AND NUCLEOTIDE ANALOGUES AS POTENTIAL ANTI-AIDS AGENTS

Redox prodrug forms of the established anti-HIV agent, DDC or 2'3'-dideoxycytidine have been synthesized. Three derivatives of DDC were obtained corresponding to addition of a dihydropyridine functionality at the cytosine amino group, at the ribose 5'-hydroxyl moiety and at both sites. All of these compounds could be converted into positively charged pyridinium salts and thence to DDC itself in rat brain cytosol. Pharmacological studies in rats are currently in progress to ascertain if such DDC

lung-colonizing capabilities can be developed in this way. Positive results might afford new routes for the prevention and treatment of metastases. Other means for interfering with metastasis at the various steps of the process are also being sought using murine tumor cells, such as the Lewis lung carcinoma and PMT fibrosarcoma cells. (C.M. Foltz, L.A. Liotta, R. Muschel).

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derivatives can effectively deliver DDC to the brain. (Kinjo, Greig and Torrence).

A synthesis of poly (2-fluoroadenylyl acid) has been completed and it is under evaluation as an inhibitor of human immunodeficiency virus reverse transcriptase. (Torrence and Kohda.)

#### APPLICATION OF ORGANIC CHEMISTRY TO THE UNDERSTANDING OF THE INTERFERON-INDUCED 2-5A SYSTEM

A new solid-phase synthesis of 2'5'-oligonucleotides has been applied to the preparation of several novel analogs of 2-5A. These include: 1) sequence-specific 8-bromoadenosine analogs which have the unusual property of being 2-5A-dependent endonuclease activators as 5'-monophosphates instead of the normally required 5'-triphosphates; 2) specific 3'-fluorinated ribose analogs which may be able to replace the important role of the 3'-hydroxyl moiety in activation of the 2-5A-dependent endonuclease. (Lesiak, Kovacs and Torrence.)

#### INHIBITION OF VESICULAR STOMATITIS VIRUS RNA POLYMERASE BY 2'5'-OLIGOADENYLATES

The dinucleotide tetraphosphate, ppp5'A2'p5'A, has been shown to block vesicular stomatitis virus (VSV) replication in intact HeLa cells, and other reports have suggested that interferon inhibits VSV replication at the level of primary transcription. We have found that ppp5A2'p5'A inhibits RNA polymerase activity in isolated VSV nucleocapsids. Inhibition was specific to the dimer species, but the 3',5'-isomer also could inhibit at the same concentrations. The concentrations of 2-5A dimer required to inhibit the VSV polymerase suggests that this is not likely to be an important mechanism of viral inhibition by interferon. (Lenard, Lesiak, Kovacs, Torrence.)

#### SECTION ON MEDICINAL CHEMISTRY

Work was concentrated on the biologically active alkaloids colchicine, physostigmine, beta-carbolines and oxoaporphines and has progressed to a point where synthesis of medically useful analogs of colchicine and physostigmine can be envisaged. The goal of these efforts, however, is the collection of data which will allow a better understanding of the mode of action of these alkaloids on a molecular level. Included in the program was the detection and characterization of potential metabolites of the novel antimalarial drug arteether, derived from the Chinese drug artemisinin. Progress on the origin of mammalian reticuline, a biosynthetic precursor of mammalian morphine, was made.

## COLCHICINE

Reaction of thiocolchicine with the commercially available Lawesson's reagent afforded 9-thiodeoxothiocolchicine, a representative of a new class of highly potent antitubulin compounds. Several compounds, highly potent in antitubulin assays, were prepared from 3-demethyl-thiocolchicine by esterification of the OH-group, and by replacing the NH-Ac group with a NH-CHO or a NH-COC<sub>3</sub>H<sub>7</sub> group. A novel synthesis of desamino-colchicol methyl ether has been accomplished from easily available tetramethoxy-biphenyls. Intermediates of this synthesis are useful compounds for preparing alkaloids containing medium sized rings and for studying lignans reported to have beneficial effects in liver disorders. Synthesis of 7-isothiocyanato-deacetamidocolchicine, with a deuterio-label at C(2), has been accomplished. Binding of the tritiated analog is supposed to afford, through covalent binding to tubulin, a protein-thiocolchicine complex which will be chemically degraded. (A. Brossi, O. Boye, Y. Itoh, and A. Muzaffar.)

## PHYSOSTIGMINE

New carbamate analogs of (-)-physostigmine, which are longer acting and inhibit acetylcholinesterase rather than butyrylcholinesterase, have been prepared. It is hoped that a medically useful therapeutic agent acting against Alzheimers disease and other geriatric disorders can be found. (+)-Physostigmine, the optical isomer of the natural alkaloid, which protects mice against organo-phosphate poisoning is, together with some analogs, under further study. The analgesic activity of (-)-eseroline, comparable in quality with morphine, prompted an investigation of more potent agonists and the synthesis of antagonists, by applying chemical modifications made long ago in the morphinan family of compounds. Total synthesis of (-)-physostigmine and (+)-physostigmine by improved procedures is on hand, and both seem technically feasible. (A. Brossi, Y. Sekine, Q. S. Yu.)

## MAMMALIAN ALKALOIDS

The most interesting of this class of compounds is morphine which is present in mammals in small amounts of questionable physiological importance. Nevertheless, the synthesis of mammalian morphine is of interest. It has been found that morphine originates from R-reticuline, which is also used as an intermediate for the synthesis of opioids by the poppy plant. Synthesis of mammalian reticuline concentrates on two pathways: the aldehyde and the less likely keto acid pathway, condensing dopamine with aldehydes or keto acids. We now have prepared norcocclaurine-1-carboxylic acid and derived isoquinolines and hope that O-methylation with S-adenosyl-methionine in the presence of catecholdecarboxylase (COMT) obtained from mammals will allow a distinction between the two pathways. Preliminary data favor strongly the aldehyde pathway since compounds obtained by the keto acid pathway O-methylate with COMT at C(7), and not at C(6) required for the biosynthesis of reticuline.

(A. Brossi, L. Chrisey, M. D. Rozwadowska, Y. Sekine.)

### ANTIVIRAL AGENTS

Oxoaporphine alkaloids have been claimed to have antifungal and antiviral properties which are particularly noteworthy in liriodenine and its methiodide. These alkaloids, however, are insoluble in commonly used solvents and in water. The colored and more soluble phenolic alkaloids, liriodendronine and its 2-methyl ether, were prepared from lysicamine which was obtained by total synthesis. These compounds will be tested for antiviral and antifungal activity. Heating the methiodide of lysicamine afforded a 2-methyl ether derivative, and a third compound which will be evaluated. (A. Brossi, V. Pabuccuoglu, M. D. Rozwadowska.)

### Beta-CARBOLINES

Reduction of the alkaloid harmaline with sodium borohydride afforded racemic tetrahydroharmine which was resolved with camphorsulfonic acid into optical isomers. Both the R- and the S- isomer of tetrahydroharmine were obtained in optically pure form. Optical purity was determined by HPLC-analysis of the ureas obtained with optically pure 1-phenylethylisocyanates. Optically active tetrahydroharmine racemize in the presence of acid. Complete racemization was observed after prolonged heating with 0.1N HCL. (A. Brossi, L. Chrisey.)

### OXINDOLES

Synthesis of racemic physovenine from an oxindole acetic ester has been achieved at the Institute of Organic Chemistry, Academia Sinica, China in collaboration with us. The phenolic intermediates of this series will be evaluated as analgesics. (A. Brossi, Q. S. Yu.)

### ANTIMALARIALS

The dimeric structure of a compound obtained from deoxydihydroartemisinin with tosylic acid in benzene was established by X-ray analysis. Pyrolysis of arteether afforded several products, some of which were most likely identical with metabolites of arteether obtained in vitro by fermentation with fungi, and with liver microsomal enzymes. (A. Brossi, A. Muzaffar, Q. S. Yu.)

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 58000-44 LAC

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Service Functions and Instrumentation

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D.F. Johnson	Chief, Lab. Anal. Chem.	LAC/NIDDK
OTHER:	H.J.C. Yeh	Research Chemist	LAC/NIDDK
	N. Whittaker	Chemist	LAC/NIDDK
	W. White	Biologist	LAC/NIDDK

## COOPERATING UNITS (if any)

## LAB/BRANCH

Laboratory of Analytical

## SECTION

Instrumentation

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

4.0

## PROFESSIONAL:

4.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- |   |  |   |
|---|--|---|
| <input type="checkbox"/> (a) Human subjects | <input type="checkbox"/> (b) Human tissues | <input checked="" type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors        |  |   |
| <input type="checkbox"/> (a2) Interviews    |  |   |

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Basic research and service functions are performed by members of the Section. A major mission of the organization involves the instrumental and chemical analyses provided to NIDDK scientists of the Laboratory of Chemistry, Laboratory of Bioorganic Chemistry, other NIH laboratories and to a limited extent to personnel of other government agencies. Instrumental analyses include: GC/MS spectrometry, HPLC/MS spectrometry, infrared spectrometry, nuclear magnetic resonance spectrometry, and element detection using a microwave plasma detection system. Assistance in interpretation of spectra is rendered on request. Samples of microanalysis are handled through external contracts. (D.F. Johnson, H.J.C. Yeh, N. Whittaker, W. White).

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 58001-16 LAC

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Application of NMR in Biochemical and Biological Systems

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	H. Yeh	Research Chemist	LAC/NIDDK
OTHERS:	D.M. Jerina	Sec. Chief	LBC/NIDDK
	J.M. Sayer	Research Chemist	LBC/NIDDK
	H. Yagi	Research Chemist	LBC/NIDDK
	L.K. Pannell	Visiting Scientist	LBC/NIDDK
	A. Chadha	Visiting Fellow	LBC/NIDDK
	S.K. Agarwal	Visiting Fellow	LBC/NIDDK
	A.M. Cheh	Guest Worker	LBC/NIDDK

## COOPERATING UNITS (If any)

Lab of Neuroscience, NIDDK, NIH (P. Skolnick, E. Moody)

## LAB/BRANCH

Laboratory of Analytical Chemistry

## SECTION

Instrumentation

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2

## PROFESSIONAL:

2

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The objective of this project is to develop and apply nuclear magnetic resonance for elucidating molecular structures and for studying the interactions within and between molecules in making contributions to the solution of various chemical problems.

NMR spectroscopy has been used: 1) to study the interaction between the volatile anaesthetic halothane and rat brain tissue. Results of  $^{19}\text{F}$  NMR data suggest that anaesthetics interact nonspecifically with brain tissue and do not support the concept of the existence of an unique, saturable anaesthetic site for halothane in brain; 2) to elucidate the structures of major nucleoside adducts formed from adenine, guanine and cytosine bases of DNA and the optically active bay-region epoxides derived from polycyclic aromatic hydrocarbons; and 3) to study the mechanism of glycosidic bond cleavage of nucleosides and in particular, the reverse reaction by purine nucleoside phosphorylase.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 58002-14 LAC

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Initial Intracellular Events of Steroid Hormone Action

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	S. S. Simons, Jr.,	Chief, Steroid Hormones Section	LAC/NIDDK
OTHERS:	P.M. Yen	Staff Fellow	LAC/NIDDK
	A. Cavanaugh	PRAT Fellow/Staff Fellow	LAC/NIDDK
	H. Oshima	Visiting Fellow	LAC/NIDDK
	P. Chakraborti	Visiting Associate	LAC/NIDDK
	S. Lopez	Guest Worker	LAC/NIDDK

## COOPERATING UNITS (If any)

E. Brad Thompson (Univ. of Texas, Galveston)  
 Jeffrey M. Harmon (USUMS, Bethesda)

## LAB/BRANCH

Laboratory of Analytical Chemistry

## SECTION

Steroid Hormones

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland

## TOTAL MAN-YEARS:

7.2

## PROFESSIONAL:

5.8

## OTHER:

1.4

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☐ (b) Human tissues      ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unindented type. Do not exceed the space provided.)

The objective of this project is to define the initial intracellular events of glucocorticoid hormone action, and steroid hormone action in general. These events include steroid binding to the intracellular receptor molecule, activation of the receptor-steroid complex to a DNA-binding and nuclear-binding species, and binding of activated complexes to those nuclear acceptor sites involved in the regulation of transcription of specific genes. A combination of techniques have been used to examine the first two steps for glucocorticoid steroids. Studies with the affinity label dexamethasone mesylate (Dex-Mes) and the thiol-specific reagent methyl methanethiolsulfonate (MMTS) have revealed the involvement of two thiol groups in steroid binding. Contrary to the current dogma, we found that reduced thiols are not absolutely required for binding to glucocorticoid receptors if the steric bulk of the oxidized thiols is small. We raised polyclonal antibodies against a defined epitope near the DNA-binding domain of the receptor. These antibodies recognized only activated complexes, thus providing direct evidence for a topological change in the receptor resulting from activation. Thiol reagents were also used to establish, for the first time, functional heterogeneity among activated complexes. Thus MMTS inhibited the DNA binding of one sub-class of complexes while arsenite, which is specific for vicinal dithiols, blocked the DNA binding of the other sub-class. Furthermore, one sub-class of complexes undergoes a two-step activation process in which the association of a small molecular weight (700-3000 daltons) factor is required for DNA binding. Further studies using these techniques along with molecular biology should provide new information about steroid hormone control of gene transcription at a molecular level.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 58003-16 LAC

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Development of Methods and Materials for the Study of Medical Problems.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: C.M. Foltz

Research Chemist

LAC/NIDDK

OTHERS: B. Baer

Chemist

LAC/NIDDK

COOPERATING UNITS (if any)

Lance A. Liotta and Ruth Muschel, Pathologists, Laboratory of Pathology,  
NCI

LAB/BRANCH

Laboratory of Analytical Chemistry

SECTION

Steroid Hormones

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

0.5

PROFESSIONAL:

0.2

OTHER:

0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects

☐ (b) Human tissues

☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The primary goal of this work is to contribute to the investigation and solution of basic medical problems by the application of chemical, physical and biological methods. This goal is being pursued by studies of the biology and molecular biology of murine tumor cells with emphasis on cancer metastasis. Areas of special interest are organic chemistry, biochemistry, cell biology, tissue culture, cancer biology, cancer chemotherapy and recombinant DNA methodology.

Studies are being conducted to determine whether specific gene products confer on certain tumor cells the properties required for the formation of viable metastases. NIH 3T3 cells have been transfected with constructs of several oncogenes. Transformed cells have been selected and their tumorigenic, lung-colonizing and metastatic potencies determined by subcutaneous and tail vein injections in nude mice. The correlation of these capabilities with the expression of the oncogene introduced is to be investigated.

Additional transfections of certain cell lines, e.g., those with tumorigenic but not metastatic potency and with or without lung-colonizing potency will be performed in an attempt to endow the cells with the properties necessary for metastasis. Success in this would increase our knowledge of the genetic requirements for metastasis.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 58004-22 LAC

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Histochemistry: Principles, Methods, and Applications

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	N. Feder	Medical Officer (Research)	LAC/NIDDK
OTHERS:	W. W. Stewart	Research Physicist	LAC/NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Analytical Chemistry

SECTION

Section on Biophysical Histology

INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland, 20892

TOTAL MAN-YEARS:

2

PROFESSIONAL:

2

OTHER:

CHECK APPROPRIATE BOX(ES)

- |   |  |   |
|---|--|---|
| <input type="checkbox"/> (a) Human subjects | <input type="checkbox"/> (b) Human tissues | <input checked="" type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors        |  |   |
| <input type="checkbox"/> (a2) Interviews    |  |   |

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

Studies have continued on the professional practices of biomedical scientists and on the accuracy of the scientific literature.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 58005-16 LAC

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Interferon Induction and Action. The Antiviral Activity of Nucleoside Analogs.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: P. F. Torrence Research Chemist LAC, NIDDK

Others: D. Alster National Research Service Award Fellow LAC, NIDDK  
K. Lesiak Visiting Scientist LAC, NIDDK  
T. Kovacs Visiting Fellow (from 12/87) LAC, NIDDK

## COOPERATING UNITS (if any)

B. Uznanski, Center for Drugs and Biologics, Bethesda, MD; P. Herdejiwn, Rega Institute, Leuven, Belgium (Foreign).

## LAB/BRANCH

Laboratory of Analytical Chemistry

## SECTION

Biomedical Chemistry Section

## INSTITUTE AND LOCATION

NIH, NIDDK, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.9

## PROFESSIONAL:

2.9

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Interferon-induced enzyme activities such as the oligo (2' 5') adenylyate synthetase, the 67K dalton protein kinase and oligo (2' 5') A phosphodiesterase are investigated with the goal of understanding their role in the action of interferon, the induction of interferon by double-stranded RNA and, perhaps, control of cell growth and differentiation. Analogs of the mediator of interferon action, 2-5A, are synthesized in order to define the relationship between oligonucleotide structure and binding to and activation of the 2-5A dependent endonuclease. The eventual goal is to design useful chemotherapeutic agents based on this system.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 58006-06 LAC

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Chemistry and Metabolism of Qinghaosu: A Chinese Antimalarial Drug

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: A. Bossi Visiting Scientist LAC, NIDDK

Others: Q. S. Yu Visiting Fellow LAC, NIDDK

A. Muzaffar Visiting Fellow LAC, NIDDK

## COOPERATING UNITS (if any)

Walter Reed Army Institute of Research, Washington, D. C., NIH (W. Milhous);  
Laboratory for the Structure of Matter, Department of the Navy, Washington, D.C.,  
(J. Flippen-Anderson).

## LAB/BRANCH

Laboratory of Analytical Chemistry

## SECTION

Medicinal Chemistry Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.2

## PROFESSIONAL:

0.2

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Structure of a dimer obtained from deoxydihydroartemisinin by acid catalyzed dehydration was established by X-ray analysis.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 58007-05 LAC

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Physostigmine and Analogs

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Brossi Visiting Scientist LAC, NIDDK

Others: Q. S. Yu Visiting Fellow LAC, NIDDK

Y. Sekine Volunteer LAC, NIDDK

L. Chrisey Visiting Fellow LAC, NIDDK

## COOPERATING UNITS (if any)

Cornell University Medical School, N.Y., NIH (W. Riker); NIAID, NIH (J. R. Atack and S. I. Rapoport); University of Maryland Medical School, Baltimore, MD, (E. X. Albuquerque); Lab. for the Structure of Matter, Naval Research Institute, Washington, D.C., (J. L. Flippen-Anderson).

## LAB/BRANCH

Laboratory of Analytical Chemistry

## SECTION

Medicinal Chemistry Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Novel carbamates of the (-)-physostigmine series were prepared. Alkylation of (-)-N(1)-norphysostigmine afforded several novel N(1)-substituted analogs of (-)-physostigmine. Geneserine and geneseroline, prepared from physostigmine by oxidation with peracids, are N-oxides which ring enlarge to cyclic tetrahydrooxazines under alkaline conditions. This finding was derived from an X-ray analysis of geneseroline hydrochloride, which was found to be (-)-eseroline N-oxide hydrochloride.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 58010-04 LAC

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Mammalian Alkaloids

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Brossi Visiting Scientist LAC, NIDDK

Others: M. D. Rozwadowska Visiting Associate LAC, NIDDK

Y. Sekine Visiting Worker LAC, NIDDK

L. Chrisey Visiting Fellow LAC, NIDDK

## COOPERATING UNITS (if any)

College of Pharmacy, University of Texas at Austin (C. W. Abell); Naval Research Laboratory, Washington, D.C. (J. L. Flippen-Anderson); Laboratory of Bioorganic Chemistry, NIDDK, NIH (C. Creveling).

## LAB/BRANCH

Laboratory of Analytical Chemistry

## SECTION

Medicinal Chemistry Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

1.6

## PROFESSIONAL:

1.6

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Norcoclaurine-1-carboxylic acid decomposes at physiological pH into a yellow quinone-methide. The isoquinolines expected to be formed by O-methylation with S-adenosyl-methionine in the presence of COMT have been prepared and are fully characterized. Optically active S- and R-norcoclaurine were prepared. The S-isomer is expected to be a precursor of mammalian morphine. Both optical isomers of the following isoquinolines were prepared: salsolidine, salsoline, carnegine and N-methylsalsoline. The R-isomers proved to be much more potent inhibitors of MAO B than the S-isomers.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 58011-13 LAC

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structure-Activity Relationships of Colchicinoids Based on Tubulin Binding

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Brossi Visiting Scientist LAC, NIDDK

Others: Y. Itoh Volunteer LAC, NIDDK  
O. Boye Visiting Fellow LAC, NIDDK  
A. Muzaffar Visiting Fellow LAC, NIDDK

## COOPERATING UNITS (if any)

Division of Cancer Treatment, National Cancer Institute, NIH (E. Hamel); Laboratory of Analytical Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, NIH (H. Yeh); Sackler School, University of Tel Aviv, Israel, (M. Ravid).

## LAB/BRANCH

Laboratory of Analytical Chemistry

## SECTION

Medicinal Chemistry Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

2.2

## PROFESSIONAL:

2.2

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

Esters and N-acyl analogs of the antitumor compound 3-demethylthiocolchicine were prepared and several compounds were found highly potent in antitubulin assays in vitro. Thioethers were generally found more potent than their natural oxygenated counterparts. Thioketones of thiocolchicine were prepared with Lawesson's reagent and found highly potent in antitubulin assays. Deuterated 7-isothiocyanato-deacetamidocolchicine binds well to tubulin and has now been prepared with a deuterium label at the OCH<sub>3</sub>-group at C(2). Desaminocolchicol methyl ether, prepared by a novel total synthesis, is identical with material prepared from colchicine. The 5,6-cis-olefin of the desaminocolchicol series obtained by synthesis binds extremely well to tubulin in vitro and represents a valuable model compound.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 58012-03 LAC

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Antiviral Drugs

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Brossi Visiting Scientist LAC, NIDDK

Others: V. Pabuccuoglu Visiting Fellow LAC, NIDDK

COOPERATING UNITS (# any)

School of Pharmacy, University of Mississippi, University, MS (A. Clark and C. D. Hufford).

LAB/BRANCH

Laboratory of Analytical Chemistry

SECTION

Medicinal Chemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

0.6

PROFESSIONAL:

0.6

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The phenolic oxoaporphine alkaloids liriodendronine and its 2-O-methyl ether were prepared from lysicamine by O-demethylation with pyridinium hydrobromide at 200°C and in refluxing 48% hydrobromic acid respectively. Quaternary lysicamine methiodide undergoes decomposition in refluxing acetone. Salts of phenolic oxoaporphines are converted, on treatment with pyridine-water, into highly colored quinone-methides. The solvent system pyridine-alcohol-methylene chloride proved extremely useful to investigate chemical purity of phenolic oxoaporphines by tlc-analysis. Quinonemethides could be crystallized from pyridine-water.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 58013-03 IAC

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Beta-Carbolines

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Brossi Visiting Scientist LAC, NIDDK

Others: L. Chrisey Research Fellow LAC, NIDDK

## COOPERATING UNITS (if any)

School Of Pharmacy, Dept. of Medicinal Chemistry, University of Texas at Austin  
(C. W. Abell).

## LAB/BRANCH

Laboratory of Analytical Chemistry

## SECTION

Medicinal Chemistry Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.4

## PROFESSIONAL:

0.4

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

Optically pure tetrahydroharmine was prepared by chemical resolution and optical purity was assessed by HPLC-analysis of ureas obtained with an optically active isocyanate. Optically active tetrahydroharmine racemizes on standing in acidic solution. Several anhydronium bases of beta-carbolines were prepared and are fully characterized.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 58014-02 LAC

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Analogues of Nucleic Acids and Their Components as Potential Anti-AIDS Agents

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal investigator.) (Name, title, laboratory, and institute affiliation)

PI: P. F. Torrence Research Chemist LAC, NIDDK

Others: J. Kinjo Visiting Fellow LAC, NIDDK  
K. Lesiak Visiting Fellow LAC, NIDDK

COOPERATING UNITS (if any)

FOREIGN: Rega Institute, Catholic University of Leuven, Belgium, (Dr. E. DeClercq and J. Balzarini); Nagoya City University, Japan (Dr. K. Kohda). NIH: Dr. N. Greig, NIMH; Dr. S. Wilson, NCI.

LAB/BRANCH

Laboratory of Analytical Chemistry

SECTION

Biomedical Chemistry Section

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:

1.3

PROFESSIONAL:

1.3

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

To provide a brain-directed derivative of the anti-AIDS drug DDC, a redox prodrug form of this nucleoside was prepared. Because of the existence of two sites of acylation on DDC, three different derivatives were synthesized; a mono-O-acylated form, a mono-N-acylated form, and a di(O,N) acylated analog. These compounds could be transformed by rat brain enzymes to active DDC itself. Pharmacology studies on rats are currently in progress to determine if these derivatives can deliver improved concentrations of DDC to the brain. In a separate development, the polymer, poly (2-fluoroadenylic acid) has been synthesized and is being evaluated for activity against HIV reverse transcriptase.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 58015-02 LAC

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Oxindoles

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Brossi

Visiting Scientist

LAC, NIDDK

## COOPERATING UNITS (if any)

Institute of Organic Chemistry, Academia Sinica, Shanghai, China (Dr. Q. S. Yu).

## LAB/BRANCH

Laboratory of Analytical Chemistry

## SECTION

Medicinal Chemistry Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0

## PROFESSIONAL:

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

(+)-Physovenine present in Calabar beans has been obtained in the form of a racemic mixture by synthesis from an oxindole intermediate of the physostigmine synthesis.

This project is inactive.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 58016-01 LAC

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Inhibition of Vesicular Stomatitis Virus RNA Polymerase by 2'5'-Oligoadenylates.

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: P. F. Torrence Research Chemist LAC, NIDDK

Others: K. Lesiak Visiting Scientist LAC, NIDDK

T. Kovacs Visiting Scientist LAC, NIDDK

## COOPERATING UNITS (if any)

J. Lenard, Robert Wood Johnson Medical School, WMDNJ, Piscataway, NJ

## LAB/BRANCH

Laboratory of Analytical Chemistry

## SECTION

Biomedical Chemistry Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, Maryland 20892

## TOTAL MAN-YEARS:

0.3

## PROFESSIONAL:

0.3

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The diadenylate triphosphates, ppp5'A2'p5'A and ppp5'A3'p5'A, were found to inhibit the purified RNA polymerase ("nucleocapid") complex from vesicular stomatitis virus (vsv).

## ANNUAL REPORT OF THE LABORATORY OF NEUROSCIENCE

### NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

#### Studies on the benzodiazepine/GABA receptor chloride channel complex

The benzodiazepine/GABA receptor chloride channel complex ("supramolecular complex") is an oligomeric group of proteins that contain recognition sites for many psychopharmacological agents including benzodiazepines,  $\beta$ -carbolines, barbiturates, and "cage" convulsants (such as picrotoxin). The proteins comprising this complex act in concert to regulate the activity of chloride channels that are controlled ("gated") gamma-aminobutyric acid (GABA), the principal inhibitory neurotransmitter of the vertebrate central nervous system. Studies are in progress to characterize the molecular aspects of this system, its physiological functions and possible role in disease.

Molecular Aspects      The supramolecular complex appears to be one member of a "superfamily" of ligand-gated ion (both cation and anion) channels which may include channels gated by glycine (anion), acetylcholine (cation), and glutamate (cation). While the supramolecular complex is thought to consist of two or more distinct but related proteins, neither the number nor arrangement of subunits required to form a fully functional drug and ligand-gated chloride channel is known. Furthermore, while it has been suggested that the lumen of this channel is formed by these subunits, the presence of a distinct protein that is an integral component of the channel lumen has been proposed. Both biochemical and electrophysiological evidence suggests that "cage" convulsants act at sites in or near GABA-gated chloride channels. The exact locus of action of such compounds is important in determining the molecular arrangement of the supramolecular complex as well as more precisely defining the site of action for depressants such as the barbiturates. The synthesis of the ortho, meta and para-isothiocyano derivatives of t-butylbicycloorthobenzoate (B. daCosta and K. Rice) and the description of their neurochemical properties will provide a valuable alternative means (to electrophysiology) in estimating the minimum molecular size of the GABA-gated chloride channel. The ability of this compound to specifically inhibit GABA-responsive  $^{36}\text{Cl}$  uptake in a concentration-dependent fashion complements other neurochemical data which underscores the specificity of this compound. The recent availability of a tritiated form of the high affinity, irreversible para-isothiocyano derivative should help resolve issues of the arrangement and organization of the supramolecular complex. The observations that barbiturates differentially affect the rate of dissociation of cage convulsants from sites on the GABA-gated chloride channel in the LS and SS selected mouse lines complements previous work demonstrating differences in the biophysical properties of the supramolecular complex in these mice, and should aid in defining the molecular loci responsible

for the increased sensitivity of LS to selected depressants and convulsants.

Pharmacological Aspects Previous studies from this laboratory have led to the formulation of a molecular model defining the pharmacophore for antagonist/inverse agonist action at benzodiazepine receptors. The systematic modification of a  $\beta$ -carboline (ZK 93423) with agonist actions has led to the formulation of a pharmacophore for benzodiazepine receptor agonists containing three sites of electron density and two of lipophilicity. The demonstration that a series of n-alkanols exhibit a "cut-off" effect at GABA-gated chloride channels provides further support that the supramolecular complex may mediate many of the pharmacological actions of n-alkanols, including ethanol. Whether similar phenomena are observed at other ligand-gated channels is currently under study. Characterization of benzodiazepine receptors by fluorescence provides a rapid, less costly, and environmentally sound alternative to receptor studies with radioligands. The applicability of this technique to other receptor systems is currently under investigation.

Physiological role and implications in disease Previous studies indicate that many of the neurological manifestations in two different animal models of hepatic encephalopathy (HE) can be blocked by administration of benzodiazepine receptor antagonists such as Ro 15-1788 (flumazenil). The demonstration that tissues extracts from both models inhibit radioligand binding to benzodiazepine receptors indicates the involvement of a humoral substance(s) responsible for the encephalopathy. The partial purification and characterization of these compounds strongly indicates the presence of benzodiazepine-like materials. The behavior of sixteen inbred strains of mice in two different models of anxiety demonstrated these strains could be grouped into four distinct categories based on performance. The demonstration that 70-80% of the variance is associated with genetic factors provides an important tool for both pharmacological and molecular studies to define the anatomical and neurochemical substrates of anxiety. The observation that death of cage-cohorts produces a strain-dependent suppression of selected immune function provides a means of further examining the impact of stress of immune function. Moreover, the finding that this immunosuppression was not observed with the death of contraspecific mice indicates this may be useful in developing an animal model of bereavement.

Studies on glycine-gated channels We previously demonstrated that  $Mg^{+2}$  and other substances known to interact with NMDA-gated cation channels can modulate strychnine-insensitive  $[^3H]$ glycine binding. We examined the ability of constrained glycine analogs to interact with these strychnine-insensitive glycine binding sites, and found that 1-aminocyclopropanecarboxylic acid (ACPC) is a specific, high affinity ligand for these sites. Moreover, ACPC appears to be a

partial agonist (compared to glycine) at these sites since it is not as efficacious in enhancing [3H]MK-801 binding to NMDA-gated cation channels. Since electrophysiological studies indicate that glycine may be required for (rather than merely augment) NMDA-gated channel opening, the ability of ACPC to interfere with NMDA-mediated events was examined in vivo. ACPC was found to specifically block the lethal and convulsant action of parenterally administered NMDA as well as elicit an anticonflict action in an animal model of anxiety. These findings indicate the strychnine-insensitive glycine binding sites may be a potential locus for drug design, and indicates these sites may also be involved in certain (patho)physiological processes.

Studies on "peripheral" benzodiazepine receptors Previous pharmacological and electrophysiological studies have described the presence of recognition sites for benzodiazepines in extraneuronal tissues. These sites, referred to as "peripheral" benzodiazepine receptors, are physically and pharmacologically distinct from the benzodiazepine receptors that are components of the supramolecular complex. Using a radioactive form of AHN 086 (synthesized by A.H. Newman and K. Rice), these receptors have now been covalently radiolabelled, solubilized, and isolated from rat pineal as well as kidney mitochondria. In rat pineal, a ~30 kD form of the receptor is predominant. The recent synthesis of a radiolabelled form of AHN 070 will be useful in defining the molecular architecture of these receptors.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 58501-03 LNS
PERIOD COVERED October 1, 1988 - September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Receptors for Neurotransmitters and Drugs in Brain and Peripheral Tissues		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) P.I.: P. Skolnick Chief LN, NIDDK Others: P.K. Arora Guest Worker LN, NIDDK A.S. Basile Senior Staff Fellow LN, NIDDK K. Boje Guest Worker LN, NIDDK G.E. Evoniuk LN, NIDDK E. Fride Visiting Fellow LN, NIDDK A.H. Lewin Guest Worker LN, NIDDK J.C. Marvizon Visiting Associate LN, NIDDK R.T. McCabe Guest Worker LN, NIDDK T.D. McIntyre LN, NIDDK I.A. Paul LN, NIDDK J. Schoenheimer LN, NIDDK Y. Sei LN, NIDDK R. Trullas Visiting Fellow LN, NIDDK		
COOPERATING UNITS (if any) E. Kempner, LPB, NIDDK; E.A. Jones, S. Gammal, DDB, NIDDK; K. Rice, B. DaCosta, J. Monn, LMC, NIDDK; S. Paul, CNB, NIMH; N. Ostrowski, CPB, NIMH; D. Klein, J.A. Reig, LDN, NICHD; E. Hanna, LDMI, NICHD; (Continued)		
LAB BRANCH Laboratory of Neuroscience		
SECTION Section on Neurobiology		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 209892		
TOTAL MAN-YEARS: 10.5	PROFESSIONAL: 10	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) High affinity, stereospecific recognition sites (receptors) for neurotransmitters, neuromodulators, and many clinically useful drugs have been identified in both peripheral tissues and the central nervous system. The interaction of a neurotransmitter, neuromodulator or drug with a specific recognition site initiates a series of events (for example, the opening of an ion channel or activation of an enzyme) resulting in either a physiological/behavioral response (in the case of a neurotransmitter or neuromodulator) or pharmacological effect (in the case of a drug). Furthermore, the presence of recognition sites for synthetic compounds suggests that endogenous substances may also be present that mimic (or antagonize) the effects of exogenously applied substances. Studies are in progress to characterize "recognition-effector" systems, to link novel recognition sites to effector systems, and to relate these systems to both physiological and pathological processes. Systems under study include: a) the benzodiazepine/GABA receptor chloride ionophore complex; b) the glycine-gated chloride ionophore; c) "peripheral" benzodiazepine receptors (in both peripheral tissues and the central nervous system) and d) glycine receptors linked to NMDA-gated cation channels.		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 58,502-03LNS

PERIOD COVERED  
October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Design, Sythesis & Drugs Acting on Central and Peripheral Tissues

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	K.C. Rice	Section Chief	LN-NIDDK
CoPI:	A.E. Jacobson	Research Chemist	LN-NIDDK

COOPERATING UNITS (if any)

LAB/BRANCH  
Laboratory of Neuroscience

SECTION  
Drug Dsign and Synthesis

INSTITUTE AND LOCATION  
NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
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CHECK APPROPRIATE BOX(ES)

<input type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input checked="" type="checkbox"/> (c) Neither
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project transferred to the Laboratory of Medicinal Chemistry. The new Project number is Z01 DK 59,501-03 LMC.

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 58,503-03LNS
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Design, Synthesis, and Evaluation of Medicinal Agents & Research Tools		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)		
PI:	A.E. Jacobson	Research Chemist
CoPI:	K.C. Rice	Section Chief
		LN-NIDDK
		LN-NIDDK
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory Of Neuroscience		
SECTION Drug Design and Sythesis		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)  This project transferred to the Laboratory of Medicinal Chemistry. The new project number is Z01 DK 59,502-03LMC.		

ANNUAL REPORT OF THE MOLECULAR PATHOPHYSIOLOGY BRANCH  
National Institute of Diabetes and Digestive and Kidney Diseases

The general goals of the Branch are to investigate normal and abnormal cell function at the molecular level with emphasis on transmembrane signalling by hormones, neurotransmitters, growth factors, and other first messengers acting at the cell surface. Approaches used range from molecular biologic techniques to clinical investigation in an effort to define the pathogenesis of diseases characterized by abnormal signal transduction.

Guanine nucleotide binding proteins (G-proteins) as receptor-effector couplers

A family of G-proteins functions in transmembrane signalling as receptor-effector couplers. G-proteins couple to a diverse array of receptors including those for polypeptide hormones, monoamine neurotransmitters, photons of light, chemical odorants, chemotactic factors, and certain growth factors. Effector functions regulated by G-proteins include cAMP formation, cGMP degradation, phosphoinositide breakdown, and several types of ion channel. Major areas of interest concerning G-proteins include: 1) definition of the diversity within this gene family; tissue and subcellular distribution; regulation of gene expression. 2) definition of domains on individual G-protein subunits involved in association of the subunits, attachment to cell membranes, interaction with receptor and effector domains, and possible interactions with other regulatory proteins. 3) definition of the degree and mechanism of specificity for individual G-proteins in coupling to both receptors and effectors. 4) definition of quantitative and qualitative alterations in G-proteins that result in altered signal transduction. Significant recent progress has been made in each of these areas:

1) Characterization of promoter activity of human Gi2-alpha gene- We have cloned and defined the structure of this gene previously, and now have begun an analysis of elements in the 5'flanking region that govern gene expression. Fusion of >1kb of 5'flanking region to the reporter gene, chloramphenicol acetyl transferase, followed by transfection studies have allowed us to define a minimal region of about 100 bases 5' to the major site of initiation of transcription that is required for promoter activity. This region includes a stretch of homology (23 of 24 bases) to a comparable region in the H-ras gene promoter.

2) Molecular basis for subunit association and membrane attachment of GTP-binding proteins- G-proteins are heterotrimers; alpha subunits reversibly associate with a beta/gamma complex. The holoprotein is associated with the cytoplasmic side of the plasma membrane, but the basis for membrane attachment and the domains responsible for subunit association have not been defined. It has been postulated that the beta/gamma complex anchors alpha subunits to the membrane. We have expressed alpha subunits in cos cells and find that the proteins are targeted to the cell membrane, even though alpha subunits can be shown to be expressed in great excess (>10:1) relative to beta subunits. Thus beta subunits cannot be the sole factor responsible for alpha

attachment to the plasma membrane. We have found that alpha subunits are attached to the plasma membrane via a 1-2kDa amino-terminal "stalk." By metabolic labeling of transfected cells and immunoprecipitation of expressed alpha subunits, we can show that Gi but not Gs-alpha subunits undergo a specific fatty acid acylation (myristylation). Site-directed mutagenesis of the myristylation site has been performed to evaluate the role of this covalent modification in membrane attachment..

3) Peptide antibodies as probes of G-protein function- We have raised five antisera by immunization with synthetic decapeptides corresponding to the carboxy-terminus of various G-alpha subunits. The specificity of these antisera was rigorously characterized by ELISA, and immunoblot using defined alpha subunits expressed in E. Coli. These antibodies are useful for immunoprecipitation and immunoaffinity purification of the cognate G-alpha subunits. The antibodies were also found to block receptor-G-protein coupling without blocking effector interaction. Indeed, the Gs-alpha specific antiserum immunoprecipitated an activated Gs-adenylyl cyclase complex. These studies show that the carboxy-terminus of G-alpha is a domain required for receptor but not effector interaction. The results also suggest that these antibodies will be useful in defining the specificity of G-protein receptor and effector interactions. We have extended these studies by showing that in human platelets which contain two forms of pertussis toxin sensitive G-protein, Gi2 and Gi3, that antibodies to the former but not the latter, specifically block alpha-2-adrenergic receptor-mediated inhibition of adenylyl cyclase.

4) G-proteins in drosophila- Homologs of vertebrate Gs, Go, and Gi have been cloned in this invertebrate. These are highly homologous (>70% amino acid sequence identity) to their mammalian counterparts. In particular, the sequences corresponding to the epitopes of several of our peptide antisera are conserved. This enabled us to use these antisera to study expression and localization of drosophila G-proteins by immunoblot, and immunohistochemistry. Our data show that Gs and Go are highly expressed in the nervous system of adult organisms, but that Gi is expressed primarily in eggs and ovary at very early stages and thus may play a key role in development. The combination of the powerful genetic studies possible in drosophila and the utility of these antisera should enable us to obtain new insights into G-protein function in growth and development of a complex multicellular organism.

5) Altered G-proteins as a cause of altered signal transduction- As critical intermediates in the signal transduction pathway, quantitative or qualitative alterations in G-proteins could have a major impact on the signalling process. We have found several examples in which G-protein changes may be important. These include 1) desensitization to the effects of adenosine in fat cells is accompanied by and may be due to increases in Gs and decreases in Gi 2) certain prolactin secreting pituitary tumors are refractory to the inhibitory effects of dopamine (acting through a D2-receptor). We find selective reduction in Go expression in such tumors compared with normal anterior pituitary or dopamine sensitive tumor lines. Treatment of rats (and humans) with estrogen is also known to increase prolactin secretion and cause dopamine resistance. We now find that estrogen treatment of rats leads to decreased Go in anterior pituitary. 3) Treatment of U-937 cells with phorbol esters leads to differentiation into monocytes. Treated cells acquire responsiveness (synthesis of IL-1) to bacterial

lipopolysaccharide (LPS) LPS response is blocked by pertussis toxin, implicating a pertussis toxin-sensitive G-protein in the transduction pathway. We find that phorbol ester treatment causes increased synthesis (mRNA and protein) of the pertussis toxin substrate Gi2-alpha, and that LPS leads to covalent modification of this protein. This suggests that Gi2 is involved in coupling of LPS response in these cells.

#### Pseudohypoparathyroidism (PHP)

PHP is a genetic disorder in which resistance to parathyroid hormone (PTH) may be associated with somatic abnormalities collectively termed Albright's hereditary osteodystrophy (AHO). We have previously shown that subjects with this form of PHP are resistant to multiple hormones that act by stimulating cAMP formation, that an approximate 50% reduction in activity of the G-protein (Gs) that couples receptors to stimulation of adenylyl cyclase is present in all tissues from affected subjects, and that subjects with PHP show reduction in steady state mRNA for the Gs-alpha subunit. Studies involving use of polymerase chain reaction to amplify genomic DNA from subjects with PHP are in progress to define the abnormality at the gene level that may lead either to reduced gene transcription or decreased mRNA stability.

#### Molecular biologic studies on the cause of parathyroid neoplasia

Parathyroid tumors (benign adenomas, hyperplasia, and carcinoma) are presumptively due to acquired (and in some cases such as MEN type I to inherited) abnormalities at the gene level. We have begun to study the molecular basis for parathyroid neoplasia by searching for rearrangements and/or deletions in genomic DNA from parathyroid tumors. We have found rearrangement of the parathyroid hormone gene in only 1 of > 35 parathyroid adenomas, but this gene abnormality may be pathogenetically relevant. In both "hyperplastic" glands from subjects with MEN I and in sporadic adenomas loss of heterozygosity for loci on chromosome 11q13 was found. The data show that tumors in MEN I are monoclonal, and that a locus on 11q13 may encode a tumor "suppressor" gene.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 59000-02 MPB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Molecular biologic studies on the cause of parathyroid neoplasia

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Spiegel, M.D., Chief, Molecular Pathophysiology Branch, NIDDK

Others: E. Friedman, M.D., Visiting Fellow, MPB, NIDDK

## COOPERATING UNITS (if any)

S. Marx, M.D. Chief, Mineral Metabolism Section, MDB, NIDDK  
G. Aurbach, M.D. Chief, MDB, NIDDK  
J. Norton, M.D. Chief, Surg. Metab. Section, Surgery Branch, NCI

## LAB/BRANCH

Molecular Pathophysiology Branch

## SECTION

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

1.0

## PROFESSIONAL:

1.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Primary hyperparathyroidism (HPT) is a common endocrine disorder that can cause significant morbidity involving the renal and skeletal systems. HPT may be due to benign neoplasia of a single parathyroid gland (adenoma), benign neoplasia involving multiple parathyroid glands (hyperplasia), and rarely, to malignant neoplasia of a parathyroid gland (carcinoma). The etiology of parathyroid neoplasia has not been defined, but clinical and epidemiologic studies indicate that hyperplasia is often due to an inherited defect (multiple endocrine neoplasia types 1 and 2), and that a history of head and neck irradiation is associated with a significantly higher risk of developing parathyroid neoplasia. As with other forms of neoplasia, parathyroid tumors are presumably due to inherited (germ-line mutation) and/or acquired (somatic mutation) defects in specific genes. Etiologic genetic defects could include inappropriate expression of transforming "oncogenes" and/or loss of expression of tumor "suppressor" genes. The availability of surgically resected parathyroid tumors allows us to search for tumor-specific genetic abnormalities that may be involved in development of parathyroid neoplasia. We have found rearrangement of the parathyroid hormone gene in only 1 of > 35 parathyroid adenomas, but this gene abnormality may be pathogenetically relevant. In both "hyperplastic" glands from subjects with MEN I and in sporadic adenomas loss of heterozygosity for loci on chromosome 11q13 was found. The data show that tumors in MEN I are monoclonal, and that a locus in 11q13 may encode a tumor "suppressor" gene.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 59001-24 MPB

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Guanine nucleotide binding proteins as receptor-effector couplers

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A. Spiegel, M.D., Chief, Molecular Pathophysiology Branch, NIDDK

Others: T. Jones, M.D., Medical Staff Fellow MPB, NIDDK

A. Carter, Ph.D. Senior Staff Fellow MPB, NIDDK

P. Goldsmith, Ph.D. Research Biologist MPB, NIDDK

W. Simonds, M.D. Senior Staff Fellow MPB, NIDDK

L. Weinstein, M.D. Medical Staff Fellow MPB, NIDDK

J. Merendino, M.D. Medical Staff Fellow MPB, NIDDK

## COOPERATING UNITS (if any)

G. Milligan, Glasgow Univ., Scotland; R. Sagi-Eisenberg, (Weizman Institute, Israel); M. Brann (NINCDS); C. Unson, (Rockefeller Univ., N.Y.); P. Backlund (NIMH); M. Forte (Vollum Inst., Oregon); and J. Codina (Baylor Univ., Houston, TX).

## LAB/BRANCH

Molecular Pathophysiology Branch

## SECTION

## INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

## TOTAL MAN-YEARS:

10.5

## PROFESSIONAL:

5

## OTHER:

5.5

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☒ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

A family of guanine nucleotide binding proteins (G-proteins) functions in transmembrane signalling as receptor-effector couplers. G-proteins couple to a diverse array of receptors including those for hormones, neurotransmitters, light, odorants, and certain growth factors. Effector functions regulated (positively and, in some instances, negatively) by G-proteins include cAMP formation, phosphoinositide breakdown, potassium and calcium channels, and cGMP degradation. We have used a variety of techniques to study the expression, distribution, regulation, structure and function of G-proteins. Our studies highlight the diversity within the G-protein family. We have purified novel G-proteins and using cloned cDNAs, defined their primary structure and distribution. We have demonstrated developmental and differentiation-dependent regulation of G-protein synthesis. Using peptide specific antibodies, in situ hybridization and northern analyses, and protein reconstitution techniques, we have defined the specificity of G-proteins in coupling to receptors and effectors. We have cloned and characterized the human gene for a G-protein to define the basis for regulation of expression. These studies provide the basis for understanding the role of G-proteins in normal signal transduction and for elucidating possible defects in G-protein structure or function as the basis for abnormal signal transduction.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 59002-24 MPB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies on pseudohypoparathyroidism and related disorders

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	A. Spiegel, M.D.	Chief, Molecular Pathophysiology Branch, NIDDK
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COOPERATING UNITS (if any)

None

LAB/BRANCH

Molecular Pathophysiology Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:

0.25

PROFESSIONAL:

0.25

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☒ (b) Human tissues      ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In 1942 Albright and his associates described the features of a new clinical syndrome "pseudohypoparathyroidism" (PHP). Patients with this disorder differ from those with idiopathic hypoparathyroidism: they show characteristic constitutional features (Albright's hereditary osteodystrophy - AHO) and do not respond to exogenous parathyroid hormone (PTH). Subsequent to the original report, patients lacking the typical somatic features of AHO but resistant to endogenous and administered PTH have been described. In PHP, UcAMP (urinary cyclic AMP) does not increase normally in response to PTH administration. This indicates that there is a defective hormone receptor-adenylate cyclase complex in this disorder. We have shown that many patients with PHP/AHO (PHP Ia) show an approximately 50% reduction in activity of Gs (the stimulatory guanine nucleotide binding protein associated with adenylyl cyclase) in membranes from multiple tissues. Gs deficiency presumably accounts for resistance to multiple hormones in such patients. Patients with PHP without AHO show normal Gs activity (PHP Ib) and resistance only to PTH, and preliminary studies suggest a PTH receptor defect in such patients. Rare patients with PHP and AHO and multiple hormone resistance show normal Gs activity. Using cloned human cDNA probes for the alpha subunit of Gs, we have shown that steady state mRNA levels from fibroblasts of subjects with PHP Ia are reduced by approximately 50% compared with normals. We are now using polymerase chain reaction to amplify genomic DNA from subjects with PHP Ia in order to define the genetic abnormality responsible for Gs deficiency.

## ANNUAL REPORT OF THE LABORATORY OF MEDICINAL CHEMISTRY

### NATIONAL INSTITUTE OF DIABETES, AND DIGESTIVE AND KIDNEY DISEASES

The major research direction of the Laboratory is the elucidation of the structure and function of neurotransmitter systems in the mammalian central nervous system (CNS) and the molecular mechanism of action of CNS active drugs. Also under investigation are peripheral signaling systems and the mechanisms through which the immune and other peripheral systems are influenced by the CNS in normal and disease states. Organic/medicinal chemistry is the foundation of the multidisciplinary approach utilized in these studies which requires synthesis of novel agonists, antagonists, imaging agents, affinity ligands and other drugs for particular applications.

The Laboratory of Medicinal Chemistry was formed this year from the former Drug Design and Synthesis Section of the Laboratory of Neuroscience. The members of this new Laboratory have made major advances in medicinal chemistry which have been widely recognized. The development of The NIH Opiate Total Synthesis is one such accomplishment. This methodology is applicable for large scale opiate synthesis from readily available raw materials, and thus could provide synthetic opiates in any quantity required by the practice of medicine, thereby freeing the United States from dependence on opium from foreign countries. The medically useful narcotic antagonists naloxone and naltrexone now derived from natural thebaine are also available by this synthetic route. Furthermore, this synthetic methodology will provide the unnatural enantiomer of any opium-derived narcotic agonist or antagonist, some of which have proved to be of great importance in biomedical research and also show promise as new therapeutic agents. Other selected contributions in the development of new drugs for CNS study include design and synthesis of the following: Irazepine, the first irreversible inhibitor of the benzodiazepine (Valium) receptor; (+)- and (-)-PCMP, the first chiral derivatives of phencyclidine (PCP); FIT, the first irreversible ligand specific for the delta opioid receptor; BIT, a valuable irreversible ligand specific for the mu opioid receptor; (+)-SUPERFIT, an extremely potent and delta opioid receptor-selective irreversible ligand, which was essential for the purification of the delta opioid receptor from NG 108-15 neuroblastoma x glioma cells to *homogeneity* by biochemical collaborators; UPHIT, the first irreversible ligand specific for kappa opioid receptors; (-)-cyclofoxy, the first drug which permitted unequivocal imaging of opioid receptors in the living brain by positron emission tomography (and is now being used in a 360 patient clinical study at NIH); Metaphit, the first electrophilic irreversible inhibitor for the PCP receptor; AHN 086, the first irreversible inhibitor of the "peripheral" benzodiazepine receptor. The first synthesis of unnatural (+)-naloxone, an extremely valuable tool for study of the mechanism of the opioid receptor-endorphin system, was also accomplished. The members of this Laboratory are also responsible for the design and synthesis of new, critically important, radioligands for autoradiography and future biochemical work, as well as significant new affinity ligands for the various opioid receptors and the phencyclidine binding site. This latter site has been implicated as an allosteric site which interacts with glutamate receptors of the N-methyl-D-aspartate type. Work in this area has already resulted in the design and synthesis of potentially useful anticonvulsants for the amelioration of epilepsy, and as antischemic agents for use in stroke patients. New affinity ligands have also been designed and synthesized for the sigma receptor, which has been implicated in the neural regulation of motor behavior.

Present work in this Laboratory is concerned with rational design and the synthesis of new, highly selective ligands for drug receptors, using all of the contemporary tools of medicinal chemistry, including computer assisted molecular modeling. Areas now under intense investigation include: (1) central opioid receptor subtypes and peripheral opioid receptors, (2) binding sites on components of the immune system which resemble central opioid receptor subtypes, (3) the mechanism of cocaine and narcotic tolerance and dependence (4)

phencyclidine (PCP) recognition sites, (5) sigma, cannabinoid (marijuana) and central and peripheral benzodiazepine receptors and (6) development of new ligands for positron emission tomography (PET) and single photon emission computed tomography (SPECT) imaging of drug receptors in the CNS of living animals and conscious humans. The multidisciplinary nature of this program requires extensive collaboration with other groups from within and outside of NIH for the purpose of discernment of the structure and function of these receptors and to ensure the practical utility of the discovered ligands provided to biological and biochemical researchers. This Laboratory now is involved in collaborative work with, among others, researchers at Brown University, the University of Alabama, the Medical College of Virginia, the University of Michigan, the Free University in Holland, the University of California (Los Angeles), Meijo University in Japan, the Nathan S. Kline Institute for Psychiatric Research in New York, the Naval Research Laboratory, the Walter Reed Army Institute of Research, the National Institute of Mental Health (ADAMHA), the Nuclear Medicine Department of the Warren Grant Magnuson Clinical Center of NIH, the National Heart, Lung and Blood Institute of NIH, the National Institute of Neurological Disorders and Stroke of NIH, G. D. Searle and Co. and the Laboratory of Neuroscience of NIDDK.

Two of our National Research Service Award Fellows, Dr. James A. Monn and Dr. Andrew Thurkauf, will be leaving the Laboratory this year for positions in the pharmaceutical industry. They will be replaced by postdoctoral Fellows from well-recognized laboratories in Holland and Japan.

The following summary describes selected advances made by the Laboratory during 1988-1989.

### Opioid Receptors.

The unequivocal identification of saturable, enantioselective, high affinity mu, delta and kappa opioid receptor subtypes in the mammalian central nervous system (CNS) has resulted from many converging lines of chemical, pharmacological and biochemical investigation. In addition, evidence continues to accumulate supporting the existence of similar receptor subtypes on components of the immune system. The structure of these subtypes and their function in modulation of the CNS and immune system are extremely important questions and are currently the subject of intense investigation in many laboratories. The significance of such studies has recently been heightened by concerns regarding the relationship of opiate induced immunosuppression to the spread of acquired immune deficiency syndrome (AIDS) in intravenous narcotic abusers. Central to the success of our past and ongoing studies of receptor subtype structure and function has been the synthesis and identification of (a) selective high affinity ligands for each subtype (b) irreversible ligands with receptor subtype specificity and (c) enantiomeric pairs of these ligands for detection of receptor mediated effects.

Absolute Stereochemistry of the Pharmacologically Active Enantiomer of the Kappa Opioid Receptor Agonist U50,488. As part of our earlier kappa receptor studies, we reported synthesis and determination of the absolute configuration of the enantiomers of the highly kappa receptor selective U50,488. The absolute configuration of the active enantiomer has been revealed as 1S,2S from our studies of in vitro opioid receptor selectivity of (-)-(1S,2S)-U50,488, (+)-(1R,2R)-U50,488, and the racemate and enantiomers of cis-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide (the cis diastereomers of U50,488) and our studies of the pharmacological activities of these drugs in rhesus monkeys. Using [<sup>3</sup>H]U69,593 to label kappa binding sites of guinea pig membranes, the apparent dissociation constants of the enantiomers of U50,488 were 0.89 nM and 299 nM, for the (S,S) and (R,R) enantiomers, respectively. The (-)-cis- and (+)-cis-diastereomers showed apparent K<sub>d</sub> values of 167 nM, and 2715 nM, respectively. Binding surface analysis of the

interaction of (-)-(1S,2S)-U50,488 with kappa binding sites labeled by [3H]bremazocine resolved two binding sites at which (-)-(1S,2S)-U50,488 showed K<sub>d</sub> values of 30 nM and 10485 nM, respectively. The (±)-cis-, (-)-cis-, and (+)-cis diastereomers of U50,488 (1 micromolar) did not inhibit [3H]bremazocine binding. Rhesus monkeys were trained to discriminate ethylketocyclazocine (EKC) and saline. All compounds tested substituted completely for EKC. The order of potency was (-)-(1S,2S)-U50,488 > (±)-U50,488 > (±)-cis diastereomer of U50,488 > (+)-(1R,2R)-U50,488. In tests of analgesia, (-)-(1S,2S)-U50,488 was 2 to 4 times more potent than (±)-U50,488, while (±)-cis diastereomer of U50,488 and (+)-(1R,2R)-U50,488 were inactive at the highest doses tested (32 mg/kg). Taken collectively, these data indicate that the pharmacologically active enantiomer of U50,488 is (-)-(1S,2S)-U50,488, and provide preliminary evidence for three subtypes of kappa binding sites in guinea pig brain.

Synthesis of [3H]U50,488. Our studies in this area have now provided methodology for tritiation of U50,488 to high specific activity for the first time. The preparation of [3H](-)-1S,2S-U50,488 required four steps from N-methylcyclohexylaziridine. The synthesis of the pharmacologically active (-)-isomer of U50,488 was accomplished through the optical resolution of the intermediate 2-[1-(3-pyrrolinyl)]-N-methylcyclohexylamine using (+)-mandelic acid followed by introduction of the 3,4-dichlorophenylacetyl function and finally reductive tritiation of the pyrrolinyl function using platinum oxide as the catalyst. Purification of the resulting material afforded [3H]U50,488 of 21 Ci/mmol and 99% radiochemical purity. This sequence of reactions also established the absolute stereochemistry of the pyrrolinyl intermediates.

Affinity Ligands Specific for Kappa Opioid Receptors In Vitro. Based on knowledge of the absolute configuration of the pharmacologically active enantiomer of U50,488, we have now designed and synthesized the first kappa opioid receptor specific acylating agent, 1S,2S-trans-2-isothiocyanato-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide. We also synthesized the 1R,2R enantiomer and studied the ability of both drugs to function as irreversible inhibitors of opioid binding in vitro. Incubation of guinea pig membranes (depleted of functional mu and delta opioid receptors) with the S,S isomer followed by washing reduced the binding of the kappa selective agonist [3H]U69,593 to 11 % of control. The effect was enantiospecific since the R,R isomer had no effect under these conditions. The S,S isomer failed to effect the binding of [3H]DADLE (delta opioid receptors) and [3H]FOXY (mu opioid receptors). However, its R,R enantiomer, although having no effect on delta binding gave a significant irreversible inhibition of mu binding, an observation which strongly emphasizes the requirement for optically pure receptor probes. When kappa receptors in guinea pig brain membranes depleted of functional mu and delta opioid receptors were assayed using [3H]bremazocine, neither enantiomer irreversibly inhibited binding. The S,S isomer had an apparent IC<sub>50</sub> of 52 ± 7.5 nM in "reversible" displacement of a single concentration of [3H]U69,593. Pretreatment of guinea pig brain membranes with 100 nM of the S,S isomer followed by Scatchard analysis of [3H]U69,593 binding revealed an increase in the K<sub>d</sub> from 4.47 ± 0.21 nM to 8.45 ± 0.41 nM without significant change in the B<sub>max</sub>. A greater increase in K<sub>d</sub> without change in B<sub>max</sub> was observed on treatment of the membranes with 300 nM of the same drug. The results confirm that irreversible inhibition of [3H]U69,593 binding after preincubation with the S,S isomer is the result of a decrease in affinity of the kappa receptors as opposed to reduction in the total number of available receptors. Furthermore, failure of this drug to affect [3H]bremazocine binding in membranes depleted of functional mu and delta receptors is further evidence for heterogeneity of kappa opioid receptors.

Affinity Ligands Specific for Kappa Opioid Receptors In Vivo. Although the S,S isomer described above was highly functional in vitro as a kappa receptor specific irreversible agent, it was not effective after intracerebroventricular (ICV) injection. We have, however,

identified the (1S,2S)-trans-2-isothiocyanato-4,5-dichlorobenzeneacetamide analog as a potent affinity ligand for acylation of kappa opioid receptors *in vivo*. This drug, which we have designated "UPHIT", and its enantiomer were synthesized in 3 steps starting from optically pure (1S,2S)-(+)-trans-2-pyrrolidinyl-N-methylcyclohexylamine and its enantiomer, respectively, thus defining their absolute stereochemistry. Binding *in vitro* of UPHIT to kappa receptors labelled by [3H]U69,593 was shown to occur with an IC<sub>50</sub> value of  $25.92 \pm 0.36$  nM, whereas IC<sub>50</sub> values of  $827.42 \pm 5.88$  nM and  $115.10 \pm 1.23$  nM were obtained for the 1R,2R-enantiomer and the racemate, respectively. ICV injection of 100 micrograms of the racemate into guinea-pig brain followed by analysis of remaining kappa binding sites 24 hours later revealed that this drug depleted 98% of the kappa receptors that bind [3H]U69,593 and 40% of the kappa receptors that bind [3H]bremazocine. Initial data indicate that the activity resides principally in the S,S enantiomer as expected. These preliminary data suggest exciting uses for these compounds in furthering our knowledge of the kappa opioid receptors.

Functional Studies of Kappa Opioid Receptors with Dynorphin A. In a study of kappa receptor function, the selective kappa antagonist norbinaltorphimine (nor-BNI) was used to examine the mechanisms underlying the hindlimb paralysis, ischemia, and neuronal injury induced in the rat by the kappa opioid agonist dynorphin A. Spinal intrathecal (i.t.) injection of nor-BNI (20 nmol) either 15 minutes or immediately before i.t. injections of 5 or 20 nmoles of dynorphin A failed to alter the dynorphin A-induced disruption of hindlimb motor function and nociceptive responsiveness. Nor-BNI also did not change the three-fold increases in cerebrospinal fluid lactate concentrations produced by 20 nmoles of dynorphin A. Neuroanatomical evaluations revealed that the cell loss, fiber degeneration, and central gray necrosis in lumbosacral spinal cords of rats treated with 20 nmoles of dynorphin A were not altered by nor-BNI (20 nmoles, i.t.). Thus, the spinal cord injury and associated neurological deficits resulting from i.t. injection of dynorphin A appear to be primarily, if not totally, attributable to its non-kappa opioid action(s).

Stereochemistry-Activity Relationships with Affinity Labels Based on Fentanyl. Previously, we reported the synthesis of the first enantiomeric pair of irreversible opioid ligands (SUPERFIT and its enantiomer) based on the extremely potent cis-(+)-3-methylfentanyl and described utilization of SUPERFIT for purification of the delta opioid receptor from NG108-15 neuroblastoma x glioma cells to homogeneity. We have continued our studies of the stereochemical requirements for irreversible inhibition of opioid receptor subtypes and synthesized the corresponding trans enantiomers, (+)-trans- and (-)-trans-3-methylfentanyl isothiocyanates. A single-crystal X-ray analysis of the 2,4,6-trinitrobenzenesulfonic acid salt of the intermediate (+)-trans-3-methyl-N-phenyl-4-piperidinamine confirmed the trans configuration and revealed the absolute configuration of this compound and that of the (+)-trans-3-methylfentanyl isothiocyanate to be 3S,4S. This enantiomer was shown to be highly potent, and about 10-fold more selective as an acylating agent than the corresponding (-)-trans isomer for the higher affinity [3H]DADL (delta) binding site in rat brain membranes. In that assay, the (+)-trans and (+)-cis enantiomers were essentially equipotent as affinity ligands, and the levo enantiomers were considerably less potent. The (+)-trans enantiomer was, thus, a potent, subtype-selective acylating agent for the delta opioid receptor *in vitro*. With membranes from NG108-15 neuroblastoma x glioma hybrid cells, containing only delta receptors, the (+)-cis isomer was found to be slightly more potent than the (+)-trans drug. Similarly, the (+)-cis material was the most effective inhibitor of adenylate cyclase in these membranes, the (+)-trans drug was weakly active, and the levo enantiomers were inactive. Only (+)-cis-3-methylfentanyl isothiocyanate was found to have antinociceptive activity.

Regulation of Immune Function by Central Opioid Receptors. An *in vivo* study has now revealed the the periaqueductal gray matter (PAG) of the mesencephalon mediates opiate-

induced immunosuppression in the rat. Microinjections of morphine into the PAG resulted in a rapid suppression of natural killer (NK) cell activity, but injections into other opiate receptor-containing neuroanatomical sites including the hypothalamus, arcuate nucleus, medial amygdala, medial thalamus and dorsal hippocampus had no effect on NK activity. The suppression of NK activity could be blocked by prior peripheral administration of the opiate antagonist naltrexone. These findings demonstrate that certain actions of opiates that produce changes in NK cell function are mediated through opiate receptors in the PAG and identify a brain region involved in opiate regulation of immune function.

Opioid Receptors on Components of the Immune System for Centrally Active Delta and Kappa Drugs. Opioid peptides have been shown to modulate various parameters of both the humoral and cellular arms of the immune system. The modulatory capacity of the peptides can often be substantially reduced in the presence of naloxone, an opioid receptor antagonist, indicating a classical ligand-receptor interaction. We have extended our studies of the action of opioids on immune function, and to characterize these effects further, we investigated the characteristics of opioid receptors on a macrophage cell line, P388d1. A delta class opioid receptor was found with an Mr of 58000. We also identified opioid receptors on MOLT-4 (T-cell) and IM-9 (B cell) cell lines as well as thymocytes and T cell- and B cell-enriched populations. Using the central (brain) kappa-selective agonist, U69,593, it was also determined that P388d1 cells possess kappa-like opioid receptors. Scatchard analysis of the binding of [3H]U-69,593 revealed a single population of sites with a dissociation constant of  $17 \pm 3$  (S.E.M.) nmol/l and a total number of binding sites of  $53.8 \pm 1.0$  (S.E.M.) fmol/million cells. Moreover, the racemic kappa-selective agonist U-50,488H was able to displace 50% of [3H]U-69,593 binding at 8.0 nmol/l, whereas other opioid ligands such as DADL (delta-selective) and [D-Ala2,N-Me-Phe4,Gly5-ol]-enkephalin (mu-selective) were ineffective displacers of [3H]U-69,593 except at high concentrations.

Involvement of Opioid Receptors in Nitrous Oxide Anesthesia. The opiate antagonist naloxone was found to block nitrous oxide analgesia in an enantioselective fashion. Using a modified hotplate test in mice, the (-)-enantiomer of naloxone (which binds to mu, delta and kappa opioid receptors and has a Kd of about 1 nM) antagonized the analgesic actions of nitrous oxide in a dose-dependent (2.5-20 mg/kg) fashion. In contrast, the (+)-enantiomer (Kd 10,000 nM) had no effect on nitrous oxide analgesia at the highest dose tested (40 mg/kg). These data strongly suggest that nitrous oxide analgesia is mediated via opiate receptors and is consistent with the hypotheses that this effect occurs either through the release of endogenous opioids or by direct physical perturbation of the opiate receptor.

Upregulation of Opioid Receptors by Morphine and Naltrexone. Previously, we demonstrated that chronic administration of morphine upregulated the lower affinity binding site for [3H]DADL without producing a detectable alteration in the higher affinity binding site for [3H]DADL. We have now tested the hypothesis that chronic administration of morphine and naltrexone up-regulated the binding sites for [3H]DADL by different mechanisms. Rats were given either morphine or naltrexone chronically. Morphine up-regulated the lower affinity site, while chronic administration of naltrexone up-regulated both the higher and lower affinity binding sites for [3H]DADL. Unlike the lower affinity binding site for [3H]DADL present in membranes prepared from rats treated with placebo pellets, the lower affinity binding sites (which were up-regulated by naltrexone and morphine) were partially (naltrexone) or completely (morphine) labile during preincubation for 60 minutes at 25°C in 50 mM Tris-HCl, pH 7.4, containing 0.4 M NaCl. These data suggest that chronic administration of morphine and naltrexone up-regulate binding sites for [3H]DADL through different mechanisms, and that the lower affinity binding sites for [3H]DADL which are up-regulated by chronic administration of morphine and naltrexone might differ biochemically from the lower affinity binding sites present in membranes treated with placebo.

Differential Regulation of Opioid Receptors by Chronic Morphine and Heroin. In an effort to gain insight into differences in the pharmacological effects of heroin and morphine, we have studied effects of these drugs on regulation of mu-opiate receptors in heroin- and morphine-dependent rats. Rats rendered dependent on heroin and morphine exhibit both qualitative and quantitative differences in the characteristics of radioligand binding to mu-opioid receptors in the central nervous system. In brain membranes prepared from control animals, [3H]dihydromorphine (DHM) binding was best described by a two-site model, while in morphine-dependent rats, [3H]DHM binding was best described by a single-site model. In contrast, [3H]DHM binding to membranes from heroin-dependent animals was best described by a two-site model, with an increased density of the high-affinity, and no change in the low-affinity population compared to controls. Furthermore, both the number of binding sites for [3H]DAGO (a ligand that selectively labels a population of high-affinity mu-opiate receptors) and the sensitivity of [3H]DHM to sodium ions was increased in heroin; but not in morphine-dependent rats. These studies demonstrate that opiate receptors are differentially regulated in heroin- and morphine-dependent animals. Such neurochemical changes in mu-opiate receptors may underlie differences in the behavioral and pharmacological profiles of heroin and morphine reported in man.

Evidence for an Opioid Receptor Complex. We have investigated the effects of DADLE, DAGO, DPDPE, (0.01-1.0 micromolar) and bremazocine (0.001-0.3 micromolar) on the electrically evoked release of radiolabeled neurotransmitters and on the dopamine (DA)-stimulated cyclic AMP efflux from superfused rat brain slices. The differential inhibitory effects of these agonists on the evoked neurotransmitter release indicate that the opioid receptors mediating presynaptic inhibition of [3H]noradrenaline (NA, cortex), [14C]acetylcholine (ACh, striatum) and [3H]DA (striatum) release represent mu, delta and kappa receptors, respectively. In agreement with this classification, preincubation (60 minutes) of the slices with our delta-opioid receptor-selective irreversible ligand, fentanyl isothiocyanate (FIT, 0.01-1 micromolar), antagonized the inhibitory effects of DADLE and DPDPE on striatal [14C]ACh release only. On the other hand, the D-1 DA receptor-stimulated cyclic AMP efflux from striatal slices appeared to be inhibited by activation of mu as well as of delta receptors. In this case, the reversible mu selective antagonist, naloxone (0.1 micromolar), fully antagonized the inhibitory effect of the mu agonist, DAGO, without changing the effect of the delta agonist DPDPE but was ineffective as an antagonist in slices pretreated with FIT (1 micromolar). The inhibitory effect of DAGO on the electrically evoked [3H]NA release was antagonized by naloxone whether or not the receptors were irreversibly blocked by FIT. These data not only further support the existence of independent presynaptic mu, delta and kappa-opioid receptors in rat brain but also strongly suggest that mu and delta receptors mediating the inhibition of DA-sensitive adenylate cyclase could share a common binding site (for naloxone and FIT) and, therefore, may represent constituents of a functional opioid receptor complex.

Other evidence for the opioid receptor complex was obtained from our studies with beta-funaltrexamine (beta-FNA), an alkylating derivative of naltrexone which is indicated by a considerable body of data to be an irreversible mu receptor antagonist. However, pretreatment of rats with beta-FNA attenuates the ability of delta antagonists and naloxone to reverse delta receptor mediated physiological effects, suggesting that physically adjacent mu and delta receptors interact in vivo. This purpose of this study was to determine which opiate receptor subtype is altered by ICV injections of beta-FNA, as well by in vitro incubations with beta-FNA, and then to examine the hypothesis that pretreatment of rats with beta-FNA increases the IC50 for naloxone at the altered binding site. The results demonstrate that beta-FNA alters the conformation of the opiate receptor complex, as evidenced by a decrease in the Bmax of the lower affinity [3H]DADL binding site and a doubling of the naloxone IC50 for displacing

[3H]DADL from this site. [3H]DAGO binding sites were not detectably altered by ICV injections of beta-FNA. These data collectively support the concept of coupling among opioid receptor subtypes.

Opioid Receptor Developmental Studies. In a study of opioid receptor development in the rat, radiolabeled human beta-endorphin was cross-linked to opioid receptors from rat brain membranes using the bifunctional reagents bis-[2-(succinimidooxycarbonyloxy)ethyl]sulfone (BSCOE) and disuccinimidyl suberate (DSS). Major radiolabeled bands migrated with Mr values of 65,000, 55,000 and 33,000, however the presence of the 55 kDa band was variable. The 65 kDa band was characterized as the mu-receptor: the binding of [125I]beta-endorphin to this band was displaced by mu-selective ligands and blocked by alkylation of the receptor by the mu-specific affinity ligand BIT but not delta-specific alkylating agent FIT. The cross-linked receptor underwent alterations in molecular weight during development. Early in development, embryonic day 18 and postnatal day 1, the [125I]beta-endorphin-labeled material migrated as a single band of molecular weight 55 kDa. By day 21 postnatally the higher molecular weight band of 65 kDa was present, as was material of 53, 47 and 43 kDa. Although the protein labeled early in development migrated with a molecular weight of 55 kDa similar to the delta receptor isolated from NG108-15 neuroblastoma-glioma cells, competition studies suggest this protein is not the delta receptor. The 65 kDa band, tentatively identified as the mu-receptor, was present in adults but not detected in neonates, despite competition binding data indicating the presence of mu-sites. The results suggest that the 55 kDa band found in the 1-day-old neonate may be an immature form of the mu-opioid receptor that undergoes posttranslational modification, perhaps glycosylation, during development.

The NIH Opiate Total Synthesis. We have extensively utilized this methodology for synthesis of unnatural enantiomers of opiates and derivatives as research tools and drugs which do not bind to classical opioid receptors and thus do not act through these sites. Unnatural (+)-naloxone was essential in the study of nitrous oxide anesthesia described above and in numerous other studies. We recently synthesized unnatural [18F](+)-cyclofoxy which has proven extremely valuable for determination of nonspecific binding of [18F](-)-cyclofoxy, a ligand we introduced earlier for PET scanning studies now in progress at NIH. A highly economical and practical process for production of 3-hydroxy-4-methoxyphenylacetic acid has been developed to provide this raw material for the NIH Opiate Total Synthesis. A patent application has been filed on the process which is also applicable to synthesis of related compounds. The process is easily applicable to laboratory synthesis of more than 1 kg/batch in nearly 90% yield and could be immediately scaled up for commercial production. This methodology affords material of greater than 98% purity and directly suitable for the opiate synthesis. This process, taken together with technology recently disclosed by others for the large scale, cost effective manufacture of 3-hydroxybenzyl alcohol, is a major advance in cost reduction of drugs prepared by the NIH Opiate Total Synthesis.

The Committee on Problems of Drug Dependence Drug Testing Program. In vitro and in vivo assays have been used to study new analgesics, and stimulants and depressants, under the auspices of the Committee on Problems of Drug Dependence, with the goal of determining their physical dependence potential and abuse liability. Data from the studies on stimulants and depressants have been utilized by the Expert Committee of the World Health Organization which is charged with the evaluation of scientific data used for drug scheduling under the Psychotropic Substances Convention.

#### Central and Peripheral Benzodiazepine Receptors.

[3H]AHN 086, an Affinity Ligand for Peripheral Benzodiazepine Receptors. We previously designed and synthesized AHN 086 as a potential irreversible inhibitor of

peripheral benzodiazepine receptors (PBR) and subsequently found that this drug (an isothiocyanato derivative of Ro 5-4864 (4'-chlorodiazepam) inhibits radioligand binding to PBR with characteristics of an irreversible (acylating) ligand. We have now found that [3H]AHN 086 labels a protein of approximately 30 kDa in the rat pineal gland determined by both SDS-polyacrylamide gel electrophoresis and gel filtration high-performance liquid chromatography of digitonin-solubilized membranes. Specific incorporation of [3H]AHN 086 into this protein was inhibited by preincubating membranes with excess AHN 086. Moreover, significant specific binding of [3H]AHN 086 was not observed in either bovine pineal gland (which does not possess high-affinity binding sites for Ro 5-4864) or ovalbumin. These findings suggest that the 30 kDa protein labeled by [3H]AHN 086 in rat pineal gland is associated with peripheral benzodiazepine receptors in this tissue.

Affinity Ligands for the Benzodiazepine Receptor Coupled GABA-Gated Chloride Ionophore. In another study, the meta- and para-isothiocyanato (NCS) derivatives of t-butylbicycloorthobenzoate (TBOB) were synthesized as irreversible ligands for the gamma aminobutyric acid (GABA) regulated chloride ionophore. Catalytic reduction of the corresponding nitro compounds, followed by treatment with thiophosgene provided the drugs. p-NCS-TBOB inhibited the binding of both [3H]TBOB and [35S]t-butylbicyclophosphorothionate (TBPS) with potencies (IC<sub>50</sub> of 61 and 23 nM, respectively) similar to the parent compound. In contrast, m-NCS-TBOB, was more than 1 order of magnitude less potent (IC<sub>50</sub> of 1588 and 149 nM, respectively). The IC<sub>50</sub> values for both isothiocyanates were strongly dependent on the tissue concentration, in a manner characteristic of irreversible inhibitors. Moreover, preincubation of tissue with these compounds, followed by extensive washing, resulted in a concentration-dependent reduction in the number of [35S]TBPS binding sites and in the apparent affinity of this radioligand. Similar effects were not observed in tissues treated in identical fashion with either TBOB or picrotoxin. Preincubation with p-NCS-TBOB at concentrations that significantly inhibit [35S]TBPS or [3H]TBOB binding did not affect radioligand binding to either benzodiazepine or GABA receptors. These findings suggest that m- and p-NCS-TBOB bind irreversibly to sites labeled by cage convulsants such as TBOB and TBPS, which are on or near GABA-gated chloride channels. p-NCS-TBOB should prove useful in determining the molecular characteristics of the benzodiazepine receptor-coupled GABA gated chloride ionophore.

#### Phencyclidine Recognition Sites.

We have studied the action of phencyclidine (PCP)-like ligands on glutamate receptors of the N-methyl-D-aspartate (NMDA) type. Phencyclidine binding sites have been implicated as allosteric sites which interact with glutamate receptors of the NMDA type. Some phencyclidine (PCP)-like compounds have recently been reported to exert a robust protective effect against neuronal degeneration in ischemia models; evidence suggests they act as antagonists against the depolarizing action of NMDA in animal brain. Other sites for interaction with PCP-like ligands in the CNS have also been found, including the dopamine uptake complex.

Conformationally Restrained Analogs of PCP. The synthesis, determination of configuration, and evaluation of two conformationally restrained analogues of PCP was achieved. Brown oxidation of cis-bicyclo[3.1.0]hexan-3-ol afforded bicyclo[3.1.0]hexan-3-one in 98% yield. Treatment of this ketone with either phenyllithium or phenylmagnesium bromide in ether at room temperature followed by solvolysis of the resulting alcohol in a mixture of trifluoroacetic acid, sodium azide, and chloroform gave a mixture of cis- and trans-3-azido-3-phenylbicyclo[3.1.0]hexanes. LAH reduction of this crude mixture of azides afforded a 1:3.5 mixture of cis- and trans-3-phenyl-3-bicyclo[3.1.0]hexylamine, respectively, in 51% overall yield from the alcohol. Separation of the mixture of amines by column chromatography followed by cyclization of each furnished the two conformationally restrained analogues of

phencyclidine (PCP), cis- and trans-3-phenyl-3-piperidinylbicyclo[3.1.0]hexane in high yield. Configurations were assigned on the basis of an X-ray crystallographic analysis of the cis isomer. Bond lengths and angles are similar to those found in PCP and its derivatives. Binding to PCP receptors as well as behavioral effects in rats was determined relative to PCP. In displacement of specifically bound [3H]TCP (1-[1-(2-thienyl)cyclohexyl]piperidine) from PCP receptors, the cis isomer was nearly equipotent and the trans isomer was one-seventh as potent as PCP. These compounds were about one-fifth as potent as PCP in displacing [3H](+)-SKF 10,047 from the sigma binding site. Calculation of their ED50 values for stereotyped behavior and ataxia indicated that they were about equipotent, and 2-3-fold less active than PCP, respectively.

In Vitro and In Vivo Effect of an Alkyl Group on the Cyclohexyl Ring of PCP. In order to study the relationship between the chemical structure of ligands for the PCP binding site and their in vitro and in vivo activity, two cis and two trans enantiomers of structurally modified PCP-like compounds, the 1-(1-phenyl-3-methylcyclohexyl)piperidines were prepared from either (R)-3- or (S)-3-methylcyclohexanone through the Bruylents reaction or a modified azide route, respectively. Separation of intermediate amines was achieved through chromatography or selective crystallization of a fumarate salt. One cis isomer had about one-third of the affinity of PCP for the PCP binding site coupled to the NMDA receptor. The other isomers were less potent. There was a 40-fold difference between the binding affinity of the cis enantiomers and a fourfold difference between the affinities of the trans enantiomers. None of the compounds antagonized the stereotypy induced by phencyclidine in the rotorod assay in mice, after intraperitoneal introduction.

Synthesis, Absolute Configuration, and Molecular Modeling Study of Etoxadrol. Etoxadrol, a potent PCP agonist structurally unlike PCP, was studied. Etoxadrol is one of eight possible optical isomers of 2-ethyl-2-phenyl-4-(2-piperidyl)-1,3-dioxolane. It was synthesized from (S,S)-1-(2-piperidyl)-1,2-ethanediol, which was obtained from cleavage of dexoadrol (S,S)-2,2-diphenyl-4-(2-piperidyl)-1,3-dioxolane). The absolute configuration of etoxadrol hydrochloride was determined to be 2S, 4S, and 6S at its three chiral centers by single-crystal X-ray analysis. Epietoxadrol, epimeric with etoxadrol at the C-2 center, was also obtained from the synthesis. This much less potent enantiomer has the 2R,4S,6S configuration. The affinity of etoxadrol to the phencyclidine binding site was found to be comparable to that of phencyclidine itself and was 35 times more potent than its epimer, epietoxadrol. Three diastereomeric mixtures were prepared that had low affinity for the phencyclidine site. In studies of the discriminative stimulus properties of these compounds, it was found that only etoxadrol substituted for the phencyclidine stimulus. With use of computer assisted molecular modeling techniques, a phencyclidine binding site model was developed that, unlike a former hypothesis based on Dreiding models, correctly predicted the higher affinity of etoxadrol and the lesser affinity of epietoxadrol for the phencyclidine site.

Blockade of Dopamine Uptake Complex by Metaphit. The long-term blockade of the dopamine uptake complex by metaphit was examined. [3H]Mazindol was used to label the dopamine uptake complex in mouse striatum. In some experiments, metaphit was present in the incubation fluid throughout the procedure; in other experiments it was eliminated by several washings and centrifugations. It was found that after removal of the metaphit by washing and centrifugation, the mazindol binding was not restored. Membranes that were pretreated with metaphit and washed had a lower density of mazindol binding sites than control membranes; the remaining mazindol sites had the same affinity for [3H]mazindol. These findings are in agreement with the previous studies on [3H]cocaine and [3H]methylphenidate binding. The following observations supported the theory that metaphit acts irreversibly and does not slowly dissociate from the mazindol recognition sites of the dopamine uptake carrier complex: 1) Metaphit did not change the off-rate of [3H]mazindol binding, arguing against an allosteric

action at a distinct site. 2) The presence of cocaine protected the mazindol binding sites from the action of metaphit, supporting binding of metaphit and mazindol to the same site. 3) Nine hours after metaphit pretreatment and removal, the degree of inhibition of mazindol binding was the same as immediately after pretreatment, consonant with an irreversible effect of metaphit. 4) The potency of metaphit in inhibiting mazindol binding was greater under slightly alkaline conditions, consistent with acylation of the mazindol sites. Furthermore, it was found that intracerebroventricular application of metaphit did not result in a decrease in the binding of [ $^3$ H]mazindol 5 hours after the administration.

Synthesis of PCP-like Compounds Useful for the Amelioration of Epileptic Seizures. The anticonvulsant properties of PCP-like compounds was examined. In one study, we structurally modified the basic PCP nucleus in an attempt to obtain compounds with enhanced anticonvulsant activity relative to their neurotoxic side effects. In summary, by modifying the basic PCP nucleus, it was found to be possible to obtain compounds with a therapeutic index (TI) for protection against MES seizures as high as 3-4. This can be compared to values of 1.6, 3.2, 6.9 and 8.1 for the commonly used anticonvulsants valproic acid, phenobarbital, phenytoin and carbamazepine. The following structure-activity rules can be deduced from the present data: (i) electron withdrawing groups ( $\text{NO}_2^-$ ) at the meta position on the aromatic ring decrease toxicity more than efficacy (so that the TI is raised), whereas electron donating groups ( $-\text{NH}_2$ ,  $-\text{OCH}_3$ ) enhance efficacy and toxicity (with no improvement in TI), (ii) substitution of a primary amino group for the piperidine ring (PCA) decreases toxicity but not efficacy; moreover, conformationally restricted analogs of PCA can be synthesized which enhance the TI further, (iii) methylation of the cyclohexane ring or modifying the ring size may also improve TI. By utilizing combinations of these modifications, it may be possible to obtain further enhancement of the TI for PCP related compounds.

PCA-type Anticonvulsants. In further work on anticonvulsants, 1-phenylcyclohexylamine (PCA), an analog of phenylclidine (PCP) in which the piperidine ring is replaced by a primary amino group, and its conformationally restricted analog 1,1-pentamethylenetetrahydroisoquinoline (PM-THIQ) were found to be potent anticonvulsants in the mouse maximal electroshock (MES) seizure test (ED<sub>50</sub> values, 7.0 and 14.3 mg/kg, respectively). At higher doses, the drugs caused motor impairment and blocked the behavioral effects and lethality of i.p. injected N-methyl-D-aspartate (NMDA). The separation in potencies in the MES seizure and motor toxicity tests of PCA and PM-THIQ contrasts with PCP which was equally potent in both tests. Several compounds that were structurally related to PM-THIQ (N-ethyl-PCA, 2-methyl-PCA, N-methyl-PM-THIQ, tetrahydroisoquinoline and benzylamine) also blocked MES seizures and caused motor impairment, but failed to show as great a separation between MES anticonvulsant activity and motor toxicity. None of the compounds protected against seizures induced by the chemoconvulsant pentylenetetrazol at doses that lacked motor toxicity. The drugs were also evaluated for their ability to displace [ $^3$ H]1-[1-(2-thienyl)-cyclohexyl]piperidine from binding to high affinity acceptor sites (presumably on NMDA receptor-coupled channels) in rat brain homogenates. The rank order of potencies in the binding assay was similar to that in the behavioral tests, except for 2-methyl-PCA which was behaviorally more potent than expected. Some of the compounds failed to displace [ $^3$ H]1-[1-(2-thienyl)cyclohexyl]piperidine yet had measurable MES anticonvulsant activity (tetrahydroisoquinoline and benzylamine), indicating that an additional mechanism distinct from blockade of NMDA receptor-channels may contribute to the antiseizure activity of this class of drugs. Of the compounds examined, PM-THIQ showed the highest relative potency in the MES anticonvulsant test compared with its motor toxicity. This drug may serve as a prototype for a new class of PCP-related anticonvulsants that have less motor toxicity than PCP.

#### Synthesis of a New PCP Recognition Site Affinity Ligand, Etoxadrol meta-Isothiocyanate.

Based on our work with etoxadrol, etoxadrol meta-isothiocyanate (2S,4S,6S-2-ethyl-2-(3-isothiocyanatophenyl)-2-piperidyl)1,3-dioxolane) was synthesized and characterized as an irreversible ligand for the PCP-binding site. It is the first chiral electrophilic affinity ligand for this site to have been described. This affinity ligand is based upon etoxadrol, a 1,3-dioxolane known to have PCP-like effects in vivo and in vitro. Etoxadrol meta-isothiocyanate was found to be four to five times more potent in vitro than metaphit (1-[1-(3-isothiocyanatophenyl)cyclohexyl]piperidine), the only previously known electrophilic affinity ligand for the PCP-binding site. The binding was shown to be highly enantioselective for etoxadrol-meta-isothiocyanate. The 2R,4R,6R-enantiomer of this compound was essentially inactive. The ability of the 2S,4S,6S-enantiomer to interact with the benzodiazepine, muscarinic, and mu opioid receptor systems was also examined, and it was found not to interact with these receptor systems. It seems likely that this new affinity ligand, like metaphit, will prove to be a valuable tool for the study of the structure and function of the PCP-binding site.

Effect on Serotonin Receptors of Metaphit, an Affinity Ligand from PCP. Our studies with metaphit, which we designed and synthesized as an affinity ligand for phencyclidine binding sites, have continued with the goal of elucidating the function of those sites. We have investigated whether metaphit, a derivative of phencyclidine (PCP) which irreversibly binds to a PCP binding sites in rat brain, blocks the PCP-induced head twitch response which is produced through serotonin<sub>2</sub> (5-HT<sub>2</sub>) receptors, and also whether metaphit depleted 5-HT<sub>2</sub> receptors. We found that metaphit decreased the intensity of PCP-induced head-twitch response and had depleted both PCP and 5-HT<sub>2</sub> receptors 24 hours after administration, but it failed to block 5-HT agonist 5-methoxy-N,N-dimethyl tryptamine-induced 5-HT<sub>1A</sub> receptor-dependent behaviors. These results reconfirmed the hypothesis that PCP and 5-HT<sub>2</sub> receptors may have very similar binding sites.

Synthesis of [14C]-MDP. We synthesized [14C]-2-methyl-3,3-diphenyl-3-propanolamine (2-MDP) for the study of the metabolism and pharmacokinetic properties of this PCP-like compound. It was prepared from [14C]-carbonyl-benzophenone. 2-MDP has been shown to have similar pharmacological activity to the dissociative anesthetic PCP, and it has been shown to substitute for PCP in discriminative stimulus tests in rat and pigeon. In vitro it has been shown to compete with [3H]PCP for PCP receptor sites in CNS tissue homogenates. 2-MDP bears little structural resemblance to PCP, or to other molecules known to have PCP-like activity. Modest structural changes in the molecule which have been made, thus far, have resulted in a considerable loss in PCP-like activity. The PCP-like activity of the compound appears to be very dependent on a specific structure, unlike PCP itself, or other PCP-like compounds which can undergo considerable molecular modification and retain, or show increased PCP-like activity.

Synthesis of [18F]ITCP. In an expansion of our interest in studying brain receptor pharmacology using positron emission tomography (PET), we prepared [18F]fluorothiencylcyclohexylpiperidine ([18F]FTCP) by [18F]fluoride displacement on a mesylate precursor. The crude products were treated with borane to aid in the removal of an elimination product. Purification of the radiopharmaceutical involves a short silica gel BOND ELUT column and subsequently reverse phase HPLC. The final product has high chemical and radiochemical purity with the radiochemical yield optimized at nearly 30 percent (corrected for decay).

Synthesis of [3H]Metaphit. Metaphit has become an invaluable tool for the study of the structure and function of the PCP binding site. The availability of tritiated metaphit would therefore provide a tool for tritium labelling of PCP and sigma receptors for further study. Synthesis of [3H]metaphit was accomplished in three steps starting from 1-[1-(3-

nitrophenyl)cyclohexyl]piperidine. Introduction of the tritium-label in 20.6% radiochemical yield was achieved in the penultimate step.

Synthesis of [ $^{11}\text{C}$ ]MK801 as a Potential PET Ligand. MK801, a PCP-like compound which has been shown to have the highest affinity for the PCP binding site, has been developed by Merck, Sharpe and Dohme for its antiischemic properties. We thought that this compound, and its derivatives, would be of value for the study of the PCP binding site, and for eventual use as a PET scanning ligand. For these purposes, we have developed the facile preparation of C5(bridgehead)-substituted analogues of ( $\pm$ )-5H-dibenzo[a,d]cyclohepten-5,10-imine via lithiation of a tert-butylformamidine precursor, and have synthesized a remarkably stable and useful alpha-iodo secondary amine. We have also succeeded in synthesizing [ $^{11}\text{C}$ ]( $\pm$ )-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine ([ $^{11}\text{C}$ ]( $\pm$ )-MK801). This was accomplished via alkylation of ( $\pm$ )-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine-N-t-butylformamidine [( $\pm$ )-5-desmethyl MK801 formamidine]. The [ $^{11}\text{C}$ ] labeling was accomplished by reaction of the anion of ( $\pm$ )-5-desmethyl MK801 formamidine, generated with s-butyllithium, and [ $^{11}\text{C}$ ]methyl iodide.

PCP Ligand for Autoradiography. In order to obtain a ligand suitable for autoradiographic study of the PCP-binding site, 4-fluoro-1-[1-(2-thienyl)-3-cyclohexenyl]piperidine was efficiently synthesized in four steps starting from cyclohexane-1,4-dione monoethylene ketal. Catalytic tritiation of this key intermediate in the final step afforded the labeled compound.

### PCP Pharmacology.

PCP-induced Hypothermia Blocked by Metaphit. The acute administration of phencyclidine (PCP) causes hypothermia in the rat. Metaphit is a derivative of PCP that has been shown to irreversibly acylate PCP receptors in vitro and in vivo and can antagonize the behavioral and electrophysiological effects of PCP in the rat. We have determined that pretreatment with metaphit blocked PCP-induced hypothermia; however, pretreatment with PCP did not affect the subsequent hypothermic response to PCP. These results indicate that the antagonism of PCP-induced hypothermia by metaphit was a specific effect and not due to PCP receptor desensitization. The effects of the acute administration of the dioxolanes dexoxadrol and levoxadrol, and of PCP on body temperature in the rat were compared in a second study. Some dioxolanes, although structurally dissimilar from PCP, produce pharmacological effects that have much in common with PCP and PCP-like drugs. PCP, as well as affecting behavior, altered body temperature in the rat. Dexoxadrol produced hyperthermia but levoxadrol did not affect body temperature. In contrast to the hyperthermic effects of dexoxadrol, PCP produced hypothermia. These findings showed that while PCP and dexoxadrol may share some similar receptor binding and behavioral characteristics, they can be differentiated on the basis of their effects on body temperature.

Audiogenic Seizures Induced by Metaphit. Metaphit was found to induce audiogenic seizures in mice. The most severe clonic/tonic seizures occur 18 to 24 hours after metaphit administration. After 48 h the incidence of the seizure episodes begin to diminish. These audiogenic seizures can be prevented by the administration of either PCP or MK-801 24 hours after metaphit and 30 minutes prior to audio stimulation. These seizures may be due to a modulation of the PCP recognition site by metaphit which results in an enhanced probability that the ion channels which respond to NMDA/PCP are open.

## Sigma Receptor Ligands.

Sigma receptors are non-dopaminergic, non-opioid receptors which bind antipsychotic drugs, as well as some of the (+)-enantiomers of the benzomorphan opioids. Studies utilizing various sigma ligands have implicated sigma receptors in neural regulation of motor behavior and modulation of transmitter release upon electrical stimulation of smooth muscle preparations. Although progress is being made in elucidating the physiological roles of sigma receptors, the biochemical systems modulated by sigma receptors are not clear.

(+)-Pentazocine, a Potent Ligand for Sigma Receptors. Among ligands which bind to sigma receptors with high affinity are 1,3-di-o-tolylguanidine (DTG), (+)-pentazocine, dextrallorphan, and haloperidol. (+)-Pentazocine was the most potent ligand tested, followed by 1,3-di-o-tolylguanidine (DTG), dextrallorphan, and haloperidol. Levallorphan had less activity than its active isomer dextrallorphan, which binds with roughly ten-fold higher affinity to sigma receptors. This rank order of potency correlated well with affinity at sigma receptors. However, haloperidol was somewhat less potent than would be predicted by binding affinity, and the dose curve for haloperidol had a different slope than that for the other ligands. Studies utilizing various sigma ligands have implicated sigma receptors in neural regulation of motor behavior and modulation of transmitter release upon electrical stimulation of smooth muscle preparations. Although progress is being made in elucidating the physiological roles of sigma receptors, the biochemical systems modulated by sigma receptors remain unclear. We have shown that sigma ligands potently block stimulation of inositol phosphate production by the muscarinic cholinergic agonist, carbachol. Rat brain synaptoneurosome were prepared, labelled with [3H]myoinositol, and assayed for production of [3H]inositol phosphate. The effect of various concentrations of sigma ligands on phosphoinositide (PI) turnover was determined. Sigma ligands had no direct effect on PI turnover, with the exception that haloperidol and (+)-pentazocine caused a slight depression of basal activity at concentrations above 100  $\mu$ M. The effect of sigma and non-sigma ligands on stimulation of PI turnover by 100  $\mu$ M carbachol was determined. Sigma ligands inhibited carbachol-stimulated PI turnover in a dose-dependent manner. Sulpiride, a dopamine receptor antagonist which lacks affinity for sigma receptors failed to produce a substantial effect on carbachol-stimulated PI turnover. (+)-Oxymorphone, (+)-morphine, (+)-naltrexone, (+)-dihydrocodeinone, and (+)-nordihydrocodeinone are (+)-opiates structurally related to (+)-pentazocine and dextrallorphan. These compounds lack affinity for sigma receptors and also failed to significantly affect agonist-stimulated PI turnover in the concentration range tested. In addition, none of the compounds tested bind to PCP receptors with high affinity, ruling out involvement of PCP receptors. These observations support the notion that the effect of these ligands on PI turnover is mediated by interaction with sigma receptors. PI turnover is an important signalling mechanism in neural tissue. Several transmitter receptors have been shown to be linked to this system in a stimulatory mode. Receptors linked in an inhibitory mode have recently been reported. The excitatory amino acid (EAA) receptor agonist, glutamate, is known to inhibit norepinephrine-stimulated PI turnover in adult rat hippocampal slices. Another EAA agonist, N-methylaspartic acid, has been shown to inhibit carbachol-, histamine-, and potassium ion-stimulated PI turnover in the hippocampal slice system. The finding that sigma ligands block agonist-stimulated PI turnover suggests that sigma receptors are members of a novel family of receptors coupled to an intracellular mechanism(s) which negatively regulates components of the phosphoinositide signalling system. Agonists at these receptors may therefore serve to modulate the actions of other transmitters.

Synthesis of [3H](+)-Pentazocine for the Study of the Structure and Function of Sigma Receptors. In order to more easily study sigma receptors, a more selective tritiated ligand was sought. Thus, tritium labeled (+)-pentazocine [3H]P of specific activity 26.6 Ci/mmol was synthesized in 3 steps starting with (+)-normetazocine of defined optical purity. [3H]P was characterized as a highly selective ligand for labeling of sigma receptors. Competition data

revealed that [3H]P could be displaced from guinea pig brain membrane preparations with a number of commonly used sigma receptor ligands. [3H]P exhibited saturable, enantioselective binding with a  $K_d$  of  $5.13 \pm 0.97$  nM and a  $B_{max}$  of  $1,146 \pm 122$  fmol/mg protein. Phencyclidine (PCP) displaced [3H]P with low affinity while MK-801 was inactive, thus indicating insignificant activity at the PCP binding site; apomorphine failed to displace [3H]P indicating lack of dopamine receptor cross reactivity. Since the affinity of [3H]P is about 6 times that of the two commonly employed sigma ligands ([3H](+)-3-PPP and [3H]DTG) and since it is more selective for sigma receptors than the benzomorphan [3H]SKF-10,047, it represents the first example of a highly selective benzomorphan based sigma receptor ligand. [3H]P should prove useful for further study of the structure and function of sigma receptors.

Synthesis of Selective Sigma Receptor Ligands Based on U50,488. Sigma binding sites have been implicated in mediation of at least some of the psychotomimetic effects of PCP-like compounds. More selective ligands with high affinity for the sigma receptor were sought for the study of this receptor system. We developed potent compounds which selectively bind to the sigma receptor by the alteration of the stereochemistry of the kappa-selective opioid agonist U50,488. Thus, 1R,2S-(+)-cis-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidiny)cyclohexyl]benzeneacetamide and its 1S,2R-enantiomer were synthesized. These compounds showed high affinity for sigma receptors and negligible affinity for kappa opioid and D2-dopamine receptors.

Affinity Ligand for the Sigma Receptor. We have found that metaphit, our affinity ligand for PCP-binding sites is useful for acylation of sigma receptors. Metaphit pretreatment of guinea pig brain membranes caused loss of PCP receptors, with 50% inhibition of [3H]TCP binding occurring at 10  $\mu$ M. Metaphit also produced inhibition of binding to sigma receptors. The concentration of metaphit required to produce 50% inhibition of [3H]DTG, [3H](+)-3-PPP, and [3H](+)-SKF 10,047 binding was 2, 10, and 50  $\mu$ M, respectively. Scatchard analysis revealed an increase in the  $K_d$  of [3H]DTG and [3H](+)-3-PPP binding sites, with no significant change in the  $B_{max}$ . The differential sensitivity of the binding of the various sigma ligands and the competitive nature of metaphit acylation suggests complex interactions of these ligands with the sigma receptor.

#### 1988-1989 Non-project Activity.

Dr. Kenner C. Rice was appointed to the Editorial Advisory Board of the Journal of Medicinal Chemistry. He continues as an elected member of the Executive Committee of the Organic Chemistry Division of the American Chemical Society (ACS) and serves as liaison with the Biotechnology Secretariat of the ACS. He was elected to the Board of the Committee of Problems on Drug Dependence (CPDD) representing the American Chemical Society and also appointed as Chairman of the CPDD Immune Function Testing Committee. Dr. Rice served as an advisor to the National Institute on Drug Abuse Technical Review on "Current Chemical and Pharmacological Advances on Drugs of Abuse Which Alter Immune Function and Their Impact Upon HIV Infection". He presented a number of invited lectures at government agencies, a pharmaceutical company and conferences. The latter included participation in a special symposium at the fall meeting of the ACS entitled "Pharmacotherapy of Addictive Disorders: What can a Chemist do about the Crisis of Substance Abuse".

Dr. Arthur E. Jacobson was reappointed as Committee on Problems of Drug Dependence for 1988-1989, and as Affiliate Professor in the Department of Pharmacology and Toxicology, Medical College of Virginia, Virginia Commonwealth University.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER <b>Z01 DK 59,501-03 LMC</b> Formerly: <b>Z01 DK 58,502-02 LN</b>
PERIOD COVERED <b>October 1, 1988 to September 30, 1989</b>		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) <b>Design and Synthesis of Drugs Acting on Central and Peripheral Tissues</b>		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <b>PI: K. C. Rice, Laboratory Chief, LMC-NIDDK</b> <b>Co PI: A. E. Jacobson, Research Chemist, LMC-NIDDK</b> <b>OTHERS: A. Reid, Biochemist, LMC-NIDDK; B.R. de Costa, Visiting Associate, LMC-NIDDK; N. A. Grayson, IRTA, LMC-NIDDK; A. Newman, Special Volunteer, LMC-NIDDK; M. Seggel, IRTA, LMC-NIDDK; R. J. Weber, Staff Fellow, LMC-NIDDK; C.-H. Kim, Staff Fellow, LMC-NIDDK; M. Mattson, Biologist, LMC-NIDDK; A. Thurkauf, Special Volunteer, LMC-NIDDK; S. Mirsadeghi, NRC Fellow, LMC-NIDDK; S. Richardson, IRTA, LMC-NIDDK; L. Band, IRTA, LMC-NIDDK; W. Williams, Microbiologist, LMC-NIDDK; H. Xu, Special Volunteer, LMC-NIDDK</b>		
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LAB/BRANCH <b>Laboratory of Medicinal Chemistry</b>		
SECTION		
INSTITUTE AND LOCATION <b>NIDDK, NIH, Bethesda, MD 20892</b>		
TOTAL MAN-YEARS: <b>1 0</b>	PROFESSIONAL: <b>8.5</b>	OTHER: <b>1.5</b>
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>           The primary goals of this program are understanding (a) the structure and function of neurotransmitter systems in the overall operation of the mammalian central nervous system (CNS), and (b) the molecular mechanism of action of drugs which act on the CNS. Also under study are the mechanism(s) through which the immune and other peripheral systems are influenced by the CNS in normal and disease states. It is now clear that many of these systems function by interaction of cell receptors with endogenous ligands, and that production of the pharmacological effects of diverse classes of drugs requires drug-receptor interaction as a first step. Many unique opportunities for dramatic advances in the understanding of these systems have been presented by these observations but exploitation of such opportunities requires highly selective drugs as probes of the systems. The multidisciplinary approach utilized in this program employs rational drug design based on structure-activity relations and molecular modeling, modern organic chemical synthesis, pharmacology, biochemistry, immunology and requires collaboration with other appropriate disciplines. Elucidation of the molecular structure and mechanism of action of the ligand-receptor systems and the molecular mechanism of action of drugs and their antagonists will provide new opportunities for the design of superior drugs for many clinical situations and new insight into disorders which are now little-understood. Areas now under active investigation include (a) central opioid receptor subtypes, (b) opioid receptors on components of the immune system, (c) mechanisms of narcotic tolerance and dependence (d) peripheral benzodiazepine receptors (e) the benzodiazepine receptor-coupled gamma aminobutyric acid gated chloride ionophore. Synthetic programs are continuing to develop new ligands for imaging brain drug receptors by positron emission tomography (PET) and single photon emission computed tomography (SPECT) scanning. The recently developed NIH Opiate Total Synthesis continues to be employed to provide previously inaccessible unnatural enantiomers of opiates and derivatives as new pharmacological agents and research tools. The recent observation that opiate receptors in the periaqueductal grey matter of rat brain mediate morphine induced immunosuppression has provided impetus for a much more detailed, ongoing study of the effects of opioids on immune function.         </p>		

<b>DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE</b> <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER Z01 DK 59,502-03 LMC Formerly: Z01 DK 58,503-02LN
PERIOD COVERED October 1, 1988 to September 30, 1989		
TITLE OF PROJECT (90 characters or less. Title must fit on one line between the borders.) Design, Synthesis and Evaluation of Medicinal Agents and Research Tools		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: A. E. Jacobson, Research Chemist, LMC-NIDDK Co PI: K. C. Rice, Laboratory Chief, LMC-NIDDK OTHERS: A. Reid, Biochemist, LMC-NIDDK; B. R. de Costa, Visiting Associate, LMC-NIDDK; N. A. Grayson, IRTA, LMC-NIDDK; M. Seggel, IRTA, LMC-NIDDK; R. J. Weber, Staff Fellow, LMC-NIDDK; P. Hillery, Special Volunteer, LMC-NIDDK; M. Mattson, Biologist, LMC-NIDDK; J. A. Monn, Special Volunteer, LMC-NIDDK; A. Thurkauf, Special Volunteer, LMC-NIDDK; W. Williams, Biologist, LMC-NIDDK; L. Band, IRTA, LMC-NIDDK; L. Radesca, Special Volunteer, LMC-NIDDK		
COOPERATING UNITS (if any) Brown University, (W. Bowen); NRL (C. George), CN-NIMH (R. Roshman); CC-NM (R. Finn, D. Kiesewetter, M. Channing), NHLBI-CH (R. Highet), UCLA Sch. Med. (R. Pechnick; N. S. Kline Inst. Psy. (M. Reith); MN-NINDS (M. A. Rogawski)		
LAB/BRANCH Laboratory of Medicinal Chemistry		
SECTION		
INSTITUTE AND LOCATION NIDDK, NIH, Bethesda, MD 20892		
TOTAL MAN-YEARS 4.0	PROFESSIONAL 3.0	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>           Studies are in progress towards the design and synthesis of new phencyclidine-like compounds which might exert protection against neuronal degeneration, and which might have anticonvulsant (anti-epileptic) effects. The design, synthesis, and evaluation of ligands which interact specifically with particular CNS receptors are essential for the elucidation of the function and mechanism of action of these receptors. Phencyclidine binding sites have been implicated as allosteric sites which interact with glutamate receptors of the N-methyl-D-aspartate (NMDA) type. Some phencyclidine (PCP)-like compounds have recently been reported to exert a protective effect against neuronal degeneration in ischemia models; evidence suggests they act as antagonists against the depolarizing action of NMDA in animal brain. We have now prepared a number of anticonvulsants based on the structure of PCP which have little affinity for the PCP binding site and less motor toxicity in vivo. Our affinity ligand for the PCP site, metaphit, has been used to study the function of this site and its interaction with the excitatory amino acids. Metaphit has also been shown to interact with the dopamine uptake complex in the mouse striatum labeled by [3H]mazindol and was found to produce a long term blockade of [3H]mazindol binding. Several lines of evidence indicate that the blockade occurs through covalent bond formation with the isothiocyanate function present in metaphit. We have now tritiated metaphit to high specific activity and the labeled material promises to be a valuable tool for further study of the NMDA receptor-coupled binding site and the dopamine uptake complex. A new and more potent affinity ligand for the PCP binding site, etoxadrol isothiocyanate, has been designed and synthesized. We have synthesized a fluorinated derivative of TCP (the 2-thienyl analog of PCP) as a potential agent for imaging NMDA coupled PCP binding sites by positron emission tomography (PET) scanning and shown that it binds substantially better than PCP to this site. This drug has been tritiated to high specific activity for in vivo autoradiographic studies and an efficient radiochemical synthesis of the [18F] labeled material has been developed. Sigma receptors are non-dopaminergic, non-opioid receptors which bind antipsychotic drugs and have been implicated in neural regulation of motor behavior and modulation of transmitter release upon electrical stimulation of smooth muscle preparations. New, potent and selective ligands for the sigma receptor were also developed.         </p>		

ANNUAL REPORT OF PHOENIX EPIDEMIOLOGY AND CLINICAL RESEARCH BRANCH  
NATIONAL INSTITUTE OF DIABETES AND DIGESTIVE AND KIDNEY DISEASES

Overview

The Phoenix Epidemiology and Clinical Research Branch performs research in the determinants, pathogenesis and natural history of non-insulin dependent diabetes and its complications, the pathogenesis and determinants of obesity, and energy and metabolism. In addition other studies of conditions which are particularly prevalent among the American Indians of the Southwest as performed and include investigations on cholelithiasis and arthritis. The Pima Indian population of the Gila River Indian Community in Arizona forms the focus of most of this work. This population is the highest reported frequency of non-insulin dependent diabetes in the world as well as a very high prevalence of obesity, gallbladder disease and several types of arthritis, including rheumatoid arthritis and ankylosing spondylitis. The nature of the Pima Indian community and the fact that long term epidemiological and clinical research studies have been performed within it for many years has resulted in a data base in which it is possible to conduct comprehensive investigations of the occurrence and determinants of these diseases and their complications. As a result of the long term follow-up it is possible to examine genetic and environmental determinants of these conditions in more than a single generation of the population. The epidemiological studies are now conducted in a special clinic in the new Hu Hu Kam Memorial Hospital, an Indian Health Service hospital centrally located on the Gila River Indian Reservation, which opened for research and clinical activities this year. This new clinic provides much improved facilities for the epidemiologic studies compared to the portable clinic which had been in use for the past 23 years. In addition the Branch operates a 27 bed clinical research unit and associated laboratories in the Phoenix Indian Medical Center. Because of the availability of these facilities on the Gila River Reservation and in the referral hospital it has been possible to perform both epidemiological studies and detailed metabolic and laboratory studies to elucidate the causes, pathogenesis and mechanisms of these diseases and their complications. The Branch activities, therefore, can be classified into two main categories, epidemiological studies of the total population of the Gila River Indian Reservation and clinical research studies directed towards well-defined subsets of this population and appropriate control groups.

The epidemiological studies are conducted by the Diabetes and Arthritis Epidemiology Section and the clinical and laboratory studies by the Clinical Diabetes and Nutrition Section of the Branch. The Biostatistics and Data Management Section, the third component of the Branch, supports data processing, data management and statistical requirements of the other sections by providing support to investigators in matters relating to data processing, data management and biostatistics.

During the past year the Diabetes and Arthritis Epidemiology Section has continued longitudinal studies of genetic and environmental risk factors for diabetes and its complications. Longitudinal studies concerning the relationship of insulin concentrations to worsening to glucose tolerance in the development of diabetes have shown that elevated insulin concentrations precede the development of both impaired glucose tolerance and non-insulin dependent diabetes and that elevated insulin levels in children are predictive of the subsequent development of diabetes. However the onset of diabetes in adolescence and young adults occurs almost exclusively when at

least one parent has diabetes, and the presence of diabetes in a parent predicts its development in offspring over and above and independently of the prediction obtained from the insulin level alone. It appears, therefore, that an inherited factor is present which is in addition to the inherited component of insulin resistance (see below), and together, these lead to the onset of non-insulin dependent diabetes at an early age.

Because of previous studies and an increasing body of evidence that non-insulin dependent diabetes among the Pima Indians is the result of a major gene, studies have now been initiated to search for the putative gene using the techniques of genetic linkage analysis. Restriction fragment length polymorphism markers whose location has already been determined are being used to search for linkage with diabetes in informative families. Two or three generation nuclear families with at least two affected members with diabetes have been identified. The lymphocytes of informative members of these families are being collected and transformed to provide lymphoblast lines that will provide a sufficient amount of DNA to permit the search for the putative gene, and ultimately permit precise identification of both the locus and structure of the gene or genes responsible for insulin resistance and non-insulin dependent diabetes in the population.

The long-term and longitudinal nature of the epidemiological study of the Pima Indian population has permitted extensive analysis to identify the determinants of the long-term severe complications of non-insulin dependent diabetes such as end-stage renal disease, coronary heart disease, amputation, and proliferative diabetic retinopathy. These studies have suggested that there is a subset of the diabetic population that is at special risk of developing one or more of these serious complications. The most clear-cut evidence of this has been obtained for end-stage renal disease and diabetic nephropathy. In families in which two generations are affected by non-insulin dependent diabetes it has been possible to determine the likelihood of the offspring developing end-stage renal disease or diabetic nephropathy according to whether or not one or more diabetic parents is similarly affected. Diabetic offspring have been shown to have a much greater risk of developing end-stage renal disease or diabetic nephropathy if a diabetic parent has been similarly affected. These findings strongly suggest that there may be independent genetic determinants of some of the complications of diabetes which are expressed only in the presence of diabetes. The availability of such multigeneration families within the Pima Indian population suggest the possibility of identifying the genetic determinants of such complications in addition to those of non-insulin dependent diabetes mellitus itself.

Investigations carried out by the Clinical Diabetes and Nutrition Section have focused in three major areas: The investigation of the pathogenesis and mechanisms of the development of diabetes; the mechanisms and biochemical basis of insulin resistance; and the role of disordered energy metabolism in the pathogenesis of obesity.

The prospective study of the pathogenesis of impaired glucose tolerance and non-insulin dependent diabetes that has been conducted over the past several years has led to important concepts concerning the pathophysiology of NIDDM. Among the subjects originally recruited and investigated in this study, 32 have now developed non-insulin dependent diabetes and many others have developed impaired glucose tolerance. It has been shown that the development of impaired glucose tolerance is accompanied by worsening of insulin resistance, increased circulating insulin concentrations and increasing obesity. The transition from impaired glucose

tolerance to non-insulin dependent diabetes occurs only in those with marked insulin resistance and is accompanied by reduced insulin secretion in response to secretagogues, but high circulating basal insulin levels are maintained. Insulin resistance as measured by the euglycemic clamp technique at maximal stimulating insulin levels appears to be the best long-range predictor of the development of NIDDM. Examination of the distribution of insulin resistance measured in this way has shown evidence of a trimodal frequency distribution suggesting that insulin resistance, which had previously been shown to be familial, may be the result of a single, major gene expressed in a co-dominant manner.

Studies of the biochemical mechanisms underlying insulin resistance have focused on elucidating the post-insulin receptor defects which may be responsible for decreased glycogen synthesis found in skeletal muscle of insulin resistant subjects. Previous studies have demonstrated decreased glycogen synthase activity in skeletal muscle in insulin resistant Pima Indians. Current studies include examination of the role of glycogen synthase phosphatase, phosphorylase phosphate activity, cyclic AMP dependent protein kinase activity, and insulin receptor tyrosine kinase activity. In addition, the regulation by insulin of other enzymes in human skeletal muscle, casein kinase 2 and S6 kinase activity are being examined and methods have been devised to assess tyrosine phosphatase activity in human skeletal muscle biopsy samples. Preliminary findings indicate that in addition to abnormal regulation of glycogen synthase activity there also appears to be dysregulation of S6 kinase activity by insulin in insulin resistant subjects. However, insulin receptor tyrosine kinase activity, which is low in Pima Indians with non-insulin dependent diabetes, is closely related to insulin levels and is actually higher in insulin resistant subjects with impaired glucose tolerance than in insulin sensitive subjects. This strongly suggests that the lesion that leads to insulin resistance is distal to the site of insulin receptor tyrosine kinase action but proximal to the site of glycogen synthase activation and S6 kinase activation. Additional studies to define the exact biochemical lesion responsible for the defect in insulin activation of several metabolic pathways in human skeletal muscle will be conducted.

Investigations of the determinants and pathogenesis of obesity have focused on a number of different aspects of energy metabolism. Previous work demonstrated that individuals with lower metabolic rates were at greatest risk of subsequent weight gain over a several year period. Further investigations have demonstrated that differences in respiratory quotient represent an additional risk factor for weight gain and evidence that the preferential utilization of carbohydrate or conversely a low rate of lipid oxidation - that are reflected in a change in respiratory quotient - represent an additional risk factor for the development of obesity.

Until recently it has been difficult to assess the role of physical activity in energy metabolism and the pathogenesis of obesity. Now using a doubly-labelled water technique, it is possible to measure energy expenditure under free-living conditions. This method has been implemented and carefully validated against the accurate energy expenditure measurements obtained from the respiratory chamber. The doubly-labelled water technique has been shown to be an accurate alternative method of measuring energy metabolism. New studies have been planned to use this technique to determine the effect of physical activity on energy metabolism in the free-living state.

Members of the Branch have continued to be widely sought as invited speakers at many national and international meetings on various aspects of research in diabetes and obesity. Four members of staff were invited speakers at the triennial

meeting of the International Diabetes Federation and its satellite meetings. Dr. Bogardus, Chief of the CDNS recently received the Outstanding Service Medal of the Public Health Service for his innovative and outstanding work concerning the role of insulin resistance in the development of non-insulin dependent diabetes. Two members of the Branch were selected as recipients of the American Diabetes Association Mentor Awards. Each will be able to select a postdoctoral fellow for a period of three years who will be supported entirely by the American Diabetes Association.

The Branch continues as the WHO Collaborating Centre for the Design, Methodology and Analysis of Epidemiological and Clinical Research in Diabetes. As a WHO Collaborating Centre the Branch has served as the central laboratory for the follow-up phase of the WHO Multinational Study of Vascular Disease in Diabetics as well as being a participating centre in this study. In addition the Branch has continued to advise on an intervention study to determine whether or not the progression from impaired glucose tolerance to non-insulin dependent diabetes among Chinese can be prevented through diet, exercise or a combination of these treatments. Planning has also continued for a multicenter intervention trial to determine if progression from IGT to diabetes can be prevented by dietary or drug intervention. If approved and funded this study will involve centres in Sweden, the United Kingdom, the United States and possibly elsewhere.

#### Diabetes and Arthritis Epidemiology Section

The Diabetes and Arthritis Epidemiology Section has continued the longitudinal studies of genetic and environmental risk factors for diabetes and vascular complications of diabetes in Pima Indians, as well as continuing epidemiological studies of rheumatoid arthritis, ankylosing spondylitis, osteoarthritis, cholelithiasis, mortality rates and causes of death. The long follow-up provided by this study is yielding increasingly valuable data on late complications of diabetes and the transmission of risk factors for diabetes and its complications from one generation to the next. Susceptibility to diabetes appears to be transmitted by a major autosomal gene, the location of which will be sought by means of linkage analysis. To this end the laboratory has implemented a procedure for EBV transformation of lymphocytes from members of informative pedigrees. DNA from these cells is being studied using restriction fragment length polymorphisms for purposes of linkage analysis.

Further studies have been performed on the relationship of serum insulin concentrations to worsening of glucose tolerance and the development of diabetes. Among nondiabetic children, high fasting insulin concentrations predict the development of diabetes during the subsequent 10 years, but only in children with at least one diabetic parent. This suggests that the combination of insulin resistance and some other inherited factor causes diabetes with onset in childhood or early adulthood. Longitudinal data from subjects deteriorating through the stages of normal glucose tolerance to impaired glucose tolerance (IGT) to diabetes indicate that fasting insulin concentrations increase to the stage of IGT and then change little, whereas post-load insulin increases to the stages of IGT or early diabetes and subsequently declines. These data, along with previous findings that in IGT, lower insulin responses to oral glucose predict worsening to diabetes, suggest that IGT results from insulin resistance that is partially compensated by high serum insulin concentrations, but with insulin secretory failure, persons with IGT worsen to diabetes. In diabetes, the ratio of proinsulin to total immunoreactive insulin is higher than in persons with normal glucose tolerance or IGT, consistent with the hypotheses that insulin secretion is abnormal in diabetes but not in compensated IGT.

Diabetes complications are being documented and their risk factors determined. Major complications of diabetes under study are nephropathy, end stage renal disease, retinopathy, peripheral vascular disease, and periodontal disease, all of which are related to the duration and severity of hyperglycemia and appear to develop at least as frequently in this population with non-insulin dependent diabetes as in people with insulin-dependent diabetes.

Section staff continue to be active in medical research and education beyond the projects described here. Staff collaborate extensively in research projects conducted by the Clinical Diabetes and Nutrition Section of the Branch and the National Center for Health Statistics as well as lecturing at universities and contributing to state, national, and international meetings and workshops.

#### Biostatistics and Data Management Section

The BDMS has been engaged in data management and support activities for the research operations of the Branch as a whole. The major activity is updating, error checking, storage, and retrieval of datasets for the extensive epidemiological study, as well as assistance with many smaller datasets from the studies conducted by CDNS.

Staff of the section have also been involved in analysis and organization of other complex data systems, such as that resulting from the 24 hour energy expenditure measurements made in the environmental chamber. Other major activities include support of laboratory instrument-computer interfacing, adaptation of genetics programs written by non-NIH scientists, and extensive support of personal computers.

The use of personal computers is being expanded so that many jobs can now be done more easily, at lower cost, or with greater accuracy. New applications include the use of computer graphics for most scientific presentations and publications and increased use of personal computers for data collection and report generation.

Consulting on statistical methods and data management for specific scientific projects has been the other major activity of the Section, on which much of the productivity of the direct research activities of the Branch depends.

#### Clinical Diabetes and Nutrition Section

Research at the Clinical Diabetes and Nutrition Section has been in two major areas: non-insulin dependent diabetes mellitus and obesity.

##### Pathogenesis of non-insulin dependent diabetes mellitus

Pima Indians of the Gila River Indian Community have the highest reported prevalence and incidence rate of non-insulin dependent diabetes mellitus of any population in the world: The major research effort of our section has been studying a subset of this population that is at the highest risk of developing this disease for two major purposes: 1) to determine the metabolic characteristic(s) that is most predictive of the subsequent development of non-insulin dependent diabetes mellitus among non-diabetic Pima Indians. 2) to document the sequence of metabolic events that occur during the transition from normal to impaired glucose tolerance and subsequently to the development of more severe fasting hyperglycemia and the full syndrome of non-insulin dependent diabetes mellitus. For these purposes, a large cross-sectional and longitudinal study of adult, obese offspring of Pima Indians was begun in 1982. Based on previously collected epidemiological data, approximately 30% of these offspring were expected to develop non-insulin dependent diabetes mellitus during a five-year follow-up period.

To date, approximately 300 subjects have been entered into this study and the average yearly rate of return and restudy is about 75%. To date, 32 Indians who were non-diabetic when they entered the study have developed non-insulin dependent diabetes mellitus. One of the major predictors of the development of the disease is insulin resistance, particularly at maximally insulin-stimulating concentrations. The longitudinal portions of this study are continuing to increase the accuracy of our determination of the predictors of the disease as well as to more fully characterize the metabolic changes that occur during each phase of the development of the disease. We have already observed that the deterioration of normal to impaired glucose tolerance is associated with weight gain and a worsening of insulin resistance and that the insulin secretory response to that insulin resistance appears to be appropriate to the degree of insulin resistance that develops. The subsequent transition from impaired glucose tolerance to non-insulin dependent diabetes occurs with the addition of insulin secretory failure superimposed upon previously established insulin resistance.

Among the many cross-sectional analyses that have come from this large study has been the observation of the large variability in insulin action in vivo among non-diabetics. The variance in insulin resistance is not entirely attributable to differences in degree of obesity, physical fitness, age, or gender, but appears to aggregate in families and also has a trimodal distribution. The trimodal distribution is consistent with a single-gene, autosomal, co-dominant mode of inheritance.

Thus it appears that insulin resistance may well be a major determinant of the development of non-insulin dependent diabetes mellitus in the Pima Indians and in addition that it has both a familial and likely a genetic origin. The insulin resistance is particularly manifest at maximally insulin-stimulating concentrations in vivo and therefore we have undertaken studies to determine the biochemical basis of insulin resistance in Pima Indians.

Insulin resistance, as measured by the hyperinsulinemic, euglycemic clamp technique, largely reflects insulin-mediated glucose disposal in skeletal muscle tissue. This has previously been established by combining the euglycemic clamp technique with studies of glucose uptake across the limb both by ourselves and by other investigators. In addition, using indirect calorimetry, we have established that the major pathway of insulin-mediated glucose disposal in skeletal muscle tissue is in the storage pathway or in the glycogen synthetic pathway. Thus, we focused our attention on insulin regulation of glycogen synthesis in skeletal muscle as the possible metabolic pathway most likely affected in insulin-resistant Pima Indians. Our initial work demonstrated that insulin-mediated glucose disposal rates in vivo were well correlated with insulin activation of human skeletal muscle glycogen synthase activity. Subsequent studies have therefore been addressed at determining the steps in the regulation of glycogen synthase by insulin in insulin-sensitive subjects and where that might be defective in insulin-resistant subjects.

These studies have included experiments of the glycogen synthase phosphatase activity, phosphorylase phosphatase activity, cyclic AMP-dependent protein kinase activity, and insulin receptor tyrosine kinase activity. In addition, to assess insulin regulation of other enzymes in human skeletal muscle, we have determined the insulin regulation of casein kinase II and S6 kinase activity and developed the methodologies to assess tyrosine phosphatase activity in human skeletal muscle biopsy samples. These studies to date have shown that in addition to abnormal insulin regulation of glycogen synthase in insulin-resistant subjects, there also appears to be a dysregulation of S6 kinase activity by insulin in insulin-resistant subjects so that the metabolic chemical lesion at the origin of the insulin resistance must be common to both the glycogen synthase activation as well as the S6 kinase activation. A possible mechanism of the insulin dysregulation of glycogen synthase in insulin-resistant subjects was that there was a deficient insulin activation of glycogen synthase phosphatase in insulin-resistant subjects compared to insulin-sensitive

subjects and this in turn was possibly related to a dysregulation of cyclic AMP-dependent protein kinase activity by insulin in these insulin-resistant individuals. Despite these several abnormalities of insulin action in human skeletal muscle that are post insulin receptor, there were no abnormalities of insulin regulation of the tyrosine activity of the insulin receptor observed in insulin-resistant subjects compared to insulin-sensitive subjects. Further experiments are planned to continue to define the exact biochemical lesion responsible for the deficient insulin activation of several metabolic pathways in human skeletal muscle in insulin-resistant Pima Indians.

### Obesity

The Pima Indians have an extraordinarily high prevalence of obesity and the obesity is a major risk factor for the development of non-insulin dependent diabetes mellitus. We have undertaken several investigations to determine whether an abnormal energy metabolism contributes to the pathogenesis of obesity in this population. In 1985, a human respiratory chamber was constructed at the Clinical Diabetes and Nutrition Section to study human energy expenditure and substrate utilization rates over a 24-hour period. This would enable us to study the different components of 24-hour energy expenditure - the sleeping metabolic rate, resting metabolic rate, thermic response to food, and the energy cost of physical activity. In addition, the technique of indirect calorimetry permits the determination of substrate utilization rates, based on the respiratory quotient, over a 24-hour period.

Over the past few years, several hundred Pima Indians as well as Caucasians have been studied in our respiratory chamber during our longitudinal study of a subset of the population. The major findings to date have been that the resting metabolic rate as well as the 24-hour metabolic rate are familial characteristics in the population, independent of the familial aggregation of body size and age. Thus the metabolic rate varies to some degree between individuals, possibly on a genetic basis. More importantly, however, we observed that the individuals with the lowest metabolic rates were at the greatest risk of subsequent weight gain during a several-year follow-up period. In fact, there was a correlation between the rate of body weight gain and the 24-hour metabolic rate, adjusted for individual differences in body size, age, and sex. There also appears to be significant individual variation in the 24-hour substrate utilization rates even while eating diets of the same composition. The variation in the substrate utilization rates and (or respiratory quotient) were not entirely explained by individual differences in degree of obesity, sex, physical activity, or age. Furthermore, a high respiratory quotient or a high rate of carbohydrate oxidation, or conversely a low rate of lipid oxidation, appear to be an additional risk factor for weight gain in the population - and this was independent and in addition to the risk of weight gain imparted due to a low metabolic rate. However, there have been no clear relationships between the degree of physical activity and subsequent weight gain in those individuals studied to date. This may be because the studies are conducted in the confined conditions of the respiratory chamber.

To more appropriately assess the contribution of physical activity to changes in weight, we are developing the doubly-labeled water method of measuring energy expenditure in the free-living condition. This method makes use of a double stable isotope of water - deuterium and  $^{18}\text{O}$  - to measure carbon dioxide production and thereby energy expenditure in free-living conditions. Our preliminary studies suggest that the doubly-labeled water method is quite comparable to rates of energy expenditure measured in the respiratory chamber and should permit us to, by measuring energy expenditure both in the chamber and in the free-living condition, assess more fully the contribution made by differences in physical activity to differences in weight gain over time.

Techniques are being developed to assess the contribution of muscle energy metabolism to the variability in metabolic rate in Pima Indians as well as to assess the relationship between differences in food preferences (fat versus carbohydrate, in particular) in subjects with low metabolic rates and high metabolic rates. To perform these studies, we are developing a computer-operated vending machine apparatus to allow us to assess food preferences in individuals for whom we have also made detailed studies of energy metabolism.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69000-24 PECR

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Diabetes Mellitus and Other Chronic Diseases in the Gila River Indian Community

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	W.C. Knowler	Chief	DAES, NIDDK
Others:	P.H. Bennett	Chief	PECRB, NIDDK
	D.J. Pettitt	Assistant Chief	DAES, NIDDK
	R.G. Nelson	Staff Fellow	DAES, NIDDK
	M.F. Saad	Visiting Associate	DAES, NIDDK
	D. Mott	Research Chemist	CDNS, NIDDK
	W.J. Butler	Computer Systems Analyst	BDMS, NIDDK
	H.R. Baird	Mathematician	BDMS, NIDDK

## COOPERATING UNITS (if any)

Biostat. and Data Management Sec. Clinical Diabetes and Nutrition Sec., PECRB, NIDDK; Indian Health Service; Ariz. State U.; State U. of New York at Buffalo; Cleveland Clinic, Cleveland OH

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Diabetes and Arthritis Epidemiology Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

## TOTAL MAN-YEARS:

6.7

## PROFESSIONAL:

3.7

## OTHER:

3.0

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of this project is to identify the determinants of non-insulin-dependent diabetes, various types of arthritis, and gallbladder disease, and elucidate the natural history of the diseases and their complications. Genetic and environmental risk factors for NIDDM and vascular complications of diabetes have been studied in the Pima Indians. The residents of the study area, approximately 5000 people, have participated in a longitudinal population study for the last 24 years, allowing observations of the natural history of diabetes mellitus and its complications. Risk factors for obesity, hypertension, and cholelithiasis are also studied, along with the relationships of these diseases to diabetes and their effects on mortality rates. The genetics of diabetes is studied by means of family studies and relationships of genetics markers to disease. The roles of obesity, serum insulin concentrations, impaired glucose tolerance, occupational and leisure-time physical activity and diabetes in relatives are assessed. Risk factors for the major complications of diabetes, retinopathy, nephropathy, coronary artery disease, and peripheral vascular disease are determined by longitudinal followup of diabetic subjects. Methods of ascertainment of these complications include fundus photography, measurement of urine albumin and serum creatinine concentrations, electrocardiography, and documentation of lower extremity amputations. The severity of abnormality of glucose homeostasis is assessed by measurement of plasma glucose and serum insulin concentrations during glucose tolerance tests and measurement of glycosylated hemoglobin. This study has shown diabetes to be a serious and common disease with both genetic and environmental components. Hyperinsulinemia, reflecting insulin resistance, is an early abnormality predicting diabetes. The deterioration from impaired glucose tolerance to diabetes may be precipitated by insulin secretory failure. The complications of diabetes, especially when involving the kidney, are an important cause of increased mortality.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69001-20 PE CR

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Complications and Outcome of Diabetic and Prediabetic Pregnancies

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D.J. Pettitt	Assistant Chief	DAES, NIDDK
Others:	P.H. Bennett	Chief	PECRB, NIDDK
	H.R. Baird	Mathematician	BDMS, NIDDK
	W.C. Knowler	Chief	DAES, NIDDK
	R.G. Nelson	Staff Fellow	DAES, NIDDK
	M.F. Saad	Visiting Associate	DAES, NIDDK

## COOPERATING UNITS (if any)

Indian Health Service; Biostatistics and Data Management Section, PECRB.  
Karolinska Institut, Stockholm, Sweden (B. Perrson) (Foreign)  
Mayo Clinic, Rochester, Minnesota (B.A. Kottke)

## LAB/BRANCH

Phoenix Epidemiology Clinical Research Branch

## SECTION

Diabetes and Arthritis Epidemiology Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

## TOTAL MAN-YEARS:

1.5

## PROFESSIONAL:

1.0

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Macrosomia, prematurity, perinatal mortality, and congenital malformations are more common in infants of diabetic mothers than in infants of nondiabetic mothers. Offspring of diabetic women are also at an increased risk of developing obesity and glucose intolerance during childhood and young adulthood. The purposes of the project are to identify diabetes and impaired glucose tolerance during pregnancy in women in the Gila River Indian Community, to determine the effects of abnormal glucose tolerance on outcome of the pregnancy, and to determine long term prognosis for the women and their offspring. By means of a glucose tolerance test as well as chart review, the diabetes status of every woman is determined at two-yearly intervals and during the third trimester of each pregnancy. The characteristics of women who have diabetes or impaired glucose tolerance during the pregnancy are compared to those of women who are normal during the pregnancy and subsequently develop diabetes and to those of women who remain normal. At birth, cord blood has been collected for determination of glycosylated fetal hemoglobin and proinsulin. These women and their offspring, after the age of 5 years, are followed at two-yearly intervals and glucose tolerance tests are performed which include measurements of glucose and insulin. Offspring of diabetic women have more diabetes and more obesity than offspring of nondiabetic and prediabetic women. Hand and wrist x-rays have been evaluated for bone age among the offspring of diabetic women and found to be advanced relative to the bone age in the offspring of nondiabetic and prediabetic women. The findings suggest that the intrauterine environment, in addition to being an important determinant of the development of diabetes and of obesity, is also important in determining skeletal development. Analyses are under way to see if there are differences in insulin concentrations which might account for these findings.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 69003-16 PECR

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Muscle Capillary Basement Membrane Thickness Prior to Onset of Diabetes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: P.H. Bennett Chief PECRB, NIDDK

Others: C. Bogardus Chief CDNS, NIDDK  
W.C. Knowler Chief DAES, NIDDK

COOPERATING UNITS (if any)

Department of Biology, Case Western Reserve University, Cleveland, Ohio (N.B. Rushforth) and Department of Medicine, University of California, San Francisco, California (M.D. Siperstein)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85014

TOTAL MAN-YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This project is inactive.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 69006-19 PECR

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Gila River Indian Community Autopsy and Mortality Study

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: P.H. Bennett Chief PECRB, NIDDK

Others: W.C. Knowler Chief DAES, NIDDK  
D.J. Pettitt Assistant Chief DAES, NIDDK  
M.L. Sievers Guest Researcher DAES, NIDDK  
R. Nelson Senior Staff Fellow DAES, NIDDK

COOPERATING UNITS (if any)

Pathology Department, Phoenix Indian Medical Center, Indian Health Service,  
Phoenix, Arizona

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85014

TOTAL MAN-YEARS:

0.9

PROFESSIONAL:

0.7

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The causes of death and postmortem characteristics of Pima Indians of the Gila River Indian Community are investigated so that findings in subjects with and without diabetes mellitus can be correlated with studies in living subjects. Medical records are reviewed for the determination of cause of death and for the occurrence of certain serious diseases or complications of diabetes.

The purpose of the study is to relate the outcome and cause of death to events or risk factors measured in life among Pima Indian residents of the Gila River Indian Community, particularly in relation to diabetes, cardiovascular diseases and gallbladder disease. Post-mortem examinations are obtained whenever possible on members of the Gila River Indian Community to ascertain conditions present at the time of death and ascertain cause of death as precisely as possible. In addition, death certificates and all available medical records pertaining to the subjects are obtained and reviewed in a standardized way for evidence of the complications of diabetes, vascular disease, neoplasms and other conditions, which may have been recognized prior to death. The records of the occurrence of such conditions, together with conditions recognized at autopsy, are used to determine the causes of death and incidence of complications associated with diabetes and other conditions identified initially during life by the longitudinal epidemiologic studies in the population.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 69009-24 PECR

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Natural History of Arthritis and Rheumatism in the Gila River Indian Community

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: P.H. Bennett Chief PECRB, NIDDK

Others: D.J. Pettitt Assistant Chief DAES, NIDDK  
W.C. Knowler Chief DAES, NIDDK

COOPERATING UNITS (if any)

Biostatistics and Data Management Section, PECRB  
Disease Prevention, Epidemiology and Clinical Application, NIAMS (A. DelPuente)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

Diabetes and Arthritis Epidemiology Section

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, AZ, 85014

TOTAL MAN-YEARS:

1.5

PROFESSIONAL:

0.8

OTHER:

0.7

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The development and progression of osteoarthritis, rheumatoid arthritis and ankylosing spondylitis are being determined by means of clinical, radiographic and serological examinations carried out prospectively at two-yearly intervals among adults of the Gila River Indian Community (Pima Indians) in Arizona, in conjunction with epidemiological studies of diabetes in the same community. The purpose of this investigation is to ascertain the determinants of these diseases in the population, and to identify factors which alter the natural history of progression of the disease. Host factors such as age, sex, and various gene markers including HLA and Gm, together with various potential environmental determinants, such as obesity and evidence of exposure to infectious agents, will be studied prospectively to determine their relationship to the development of these diseases. Longitudinal data have now been collected over 20 years and represent a unique data set for epidemiological studies of arthritis.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69014-12 PECR

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Lipoprotein Composition and Metabolism in Pima Indians

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: C. Bogardus Chief CDNS, NIDDK

Others: B. Swinburn Visiting Associate CDNS, NIDDK

## COOPERATING UNITS (if any)

Dept of Med., Dept of Molecular Genetics, Univ of Texas, Southwest Medical School, Dallas, TX, Indian Health Service; Dept of Med., Univ. of CA, San Diego Medical School La Jolla, CA; Dept of Med., Univ of Hiroshima Medical School (foreign)

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Clinical Diabetes and Nutrition Section

## INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS:  
0.0PROFESSIONAL:  
0.0OTHER:  
0.0

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects☐ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been terminated.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69015-07 PECR

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Cross-Sectional and Longitudinal Study of "Prediabetes" in the Pima Indians

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	C. Bogardus	Chief	CDNS, NIDDK
Others:	S. Lillioja	Visiting Scientist	CDNS, NIDDK
	D.M. Mott	Research Chemist	CDNS, NIDDK
	B. Nyomba	Visiting Associate	CDNS, NIDDK
	B. Swinburn	Visiting Associate	CDNS, NIDDK
	F. Zurlo	Visiting Fellow	CDNS, NIDDK
	I. Raz	Special Volunteer	CDNS, NIDDK
	M. Saad	Visiting Associate	DAES, NIDDK

## COOPERATING UNITS (if any)

Indian Health Service

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Clinical Diabetes and Nutrition Section

## INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

## TOTAL MAN-YEARS:

8.3

## PROFESSIONAL:

4.9

## OTHER:

3.4

## CHECK APPROPRIATE BOX(ES)

<input checked="" type="checkbox"/> (a) Human subjects	<input type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither
<input type="checkbox"/> (a1) Minors		
<input type="checkbox"/> (a2) Interviews		

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The Pima Indians of Arizona have the highest prevalence and incidence rate of non-insulin dependent diabetes mellitus (NIDDM) of any population in the world. We have been studying a subset of this population that is at highest risk of developing the disease for the purposes of 1) to determine the metabolic characteristic(s) which is most predictive of the subsequent development of NIDDM among non-diabetics and 2) to document the sequence of metabolic events that occur at the transition from normal to impaired glucose tolerance and subsequently to marked fasting hyperglycemia and NIDDM. Indian volunteers are admitted to the clinical research ward for about ten days to undergo a variety of tests to assess body composition, oral and intravenous glucose tolerance, insulin secretory dynamics, energy metabolism, and insulin action. To date the results have shown that insulin resistance is a major and significant predictor of the subsequent development of NIDDM among non-diabetic subjects. The transition from normal to impaired glucose tolerance is associated with deterioration of insulin action in vivo and the insulin response to the development of this insulin resistance appears to be appropriate for the degree of glycemia that occurs with the impaired glucose tolerance. Transition from impaired glucose tolerance to marked fasting hyperglycemia is then associated with the deterioration in insulin secretory dynamics.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69018-05 PEGR

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Lipoprotein Metabolism in Diabetes and the Effects of Therapy

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: C. Bogardus Chief CDNS, NIDDK

Others: B. Swinburn

Visiting Associate

CDNS, NIDDK

## COOPERATING UNITS (if any)

2nd Dept of Med., Univ. of Helsinki, School of Med., Helsinki, Finland (Foreign);  
Indian Health Service; Dept. of Med., Univ. of Naples, Naples, Italy (Foreign); Dept.  
of Med., Inst. San Raffaele, Milan, Italy (Foreign)

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Clinical Diabetes and Nutrition Section

## INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

## TOTAL MAN-YEARS:

0.0

## PROFESSIONAL:

0.0

## OTHER:

0.0

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been terminated.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69020-06 PECR

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (20 characters or less. Title must fit on one line between the borders.)

Regulation of Muscle Glycogen Synthase Activity &amp; Insulin-Mediated Glucose Disposal

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory and institute affiliation)

PI: C. Bogardus	Chief	CDNS, NIDDK
Others: D.M. Mott	Research Chemist	CDNS, NIDDK
B.L. Nyomba	Visiting Associate	CDNS, NIDDK
S. Lillioja	Visiting Scientist	CDNS, NIDDK
I. Raz	Special Volunteer	CDNS, NIDDK
Y. Kida	Visiting Fellow	CDNS, NIDDK

## COOPERATING UNITS (if any)

Indian Health Service; Division of Biology and Medicine, Brown University, Providence, RI (D. Brautigan)

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Clinical Diabetes and Nutrition Section

## INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

## TOTAL MAN-YEARS:

1.4

## PROFESSIONAL:

1.0

## OTHER:

0.4

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Previous studies from this unit indicate that insulin resistance is primarily caused by reduced insulin-mediated glucose storage in muscle tissue which is secondary to abnormal insulin stimulation of glycogen synthase activity. We are currently characterizing the abnormalities for regulation of human muscle glycogen synthase in insulin-resistant subjects. In insulin-resistant subjects, fasting glycogen synthase phosphatase activity is reduced and fails to show the peak insulin stimulation observed for insulin-sensitive subjects at 10 minutes. A semiquantitative technique has been established using Western blots to measure the relative amount of type one protein phosphatase in human muscle from insulin-sensitive and insulin-resistant subjects. Techniques have been established to determine which subcellular compartments contain the abnormal phosphatase activity, i.e. the cytosol or the glycogen. The apparent ED50 for cAMP activation of muscle cAMP-dependent protein kinase (CPK) is not increased in insulin-resistant subjects following insulin infusion. In contrast, sensitive subjects show a reduced apparent affinity of their CPK regulatory subunit for cAMP following insulin infusion. This results in inactivation of the kinase which should stimulate glycogen synthase activity. These results suggest that abnormal regulation of both glycogen synthase phosphatase and CPK contribute to the insulin resistance which is secondary to abnormal insulin-stimulated glycogen synthase activity.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69021-09 PECR

PERIOD COVERED  
October 1, 1988 to September 30, 1989TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Energy Expenditure in Pima Indians: Risk Factors for Body Weight Gain

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: C. Bogardus	Chief	CDNS, NIDDK
Others: E. Ravussin	Visiting Scientist	CDNS, NIDDK
F. Zurlo	Visiting Fellow	CDNS, NIDDK
R. Rising	Special Volunteer	CDNS, NIDDK
B. Swinburn	Visiting Associate	CDNS, NIDDK

COOPERATING UNITS (if any)  
Indian Health Service; Metabolic Unit, Dept. of Med., Univ. of Vermont, Burlington, VT (E. Danforth); Clinical Research Center, Rockefeller Univ., New York, NY (R. Leibel); Dept. of Med. Harvard Med. Sch., Boston, MA (J. Young)LAB/BRANCH  
Phoenix Epidemiology and Clinical Research BranchSECTION  
Clinical Diabetes and Nutrition SectionINSTITUTE AND LOCATION  
NIDDK/NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS: 1.7	PROFESSIONAL: 1.7	OTHER: 0.0
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CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The Pima Indians of Arizona have the highest reported prevalence of obesity and NIDDM, two diseases tightly linked and partially genetically determined. To investigate the possible causes of body weight gain in Pima Indians, a human respiratory chamber was built in 1985 to measure daily energy expenditure in both Pima Indians and Caucasians. The results to date have shown that: 1) metabolic rate varies more than individual differences in body size, age, and sex; 2) family membership is an important determinant of the metabolic rate and the fuel mix which is oxidized to generate energy, suggesting the importance of genetic factors; 3) part of the variability in resting metabolic rate is related to differences in forearm oxygen uptake, i.e., muscle metabolism; 4) a low 24-hour fat oxidation rate is a risk factor for body weight gain independently of a low metabolic rate; 5) since the increase in obesity has been paralleled by an increase in dietary fat, we tested the effect of a high-fat diet on energy expenditure. We found that a high-fat diet did not decrease metabolic rate, suggesting that its effect on obesity is by an increase in energy intake; 6) to check the "thrifty gene hypothesis," we tested whether insulin resistance is a predictor of weight gain. We found that high, not low, rates of insulin-mediated glucose disposal predict weight gain. Therefore, if there is a genetic trait causing insulin resistance and subsequently NIDDM, it must be separate from the genetic basis of obesity; 7) in volunteers above the age of 60, we found that most of the decrease in metabolic rate observed with age is related to a decrease in fat-free mass; 8) in a validation study of the doubly-labeled water method to measure energy expenditure, we found in 8 subjects that the doubly-labeled water method was in good agreement with energy expenditure measured in a respiratory chamber (-1±6%).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE  
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER  
Z01 DK 69024-03 PECR

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) WHO Collaborating Center for Epidemiological and Clinical Investigations in Diabetes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: P.H. Bennett Chief PECRB, NIDDK

Others: W.C. Knowler Chief DAES, NIDDK

C. Bogardus Chief CDNS, NIDDK

D.J. Pettitt Assistant Chief DAES, NIDDK

COOPERATING UNITS (if any) World Health Organization, Non-Communicable Diseases Program, Geneva, Switzerland, (Foreign), Other World Health Organization Collaborating Centers for Diabetes (Foreign), China-Japanese Friendship Hospital, Beijing, China (Pan Xiao-ren) (Foreign)

LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

SECTION

INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

TOTAL MAN-YEARS:

0.5

PROFESSIONAL:

0.3

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☒ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The WHO Collaborating Center for Design, Methodology and Analysis of epidemiological and Clinical Investigations in Diabetes was designated in 1986. The purposes of the Center are to collaborate with the World Health Organization in the implementation of the WHO/IDF action program to provide advice, consultation and collaboration with other investigators in the design, methodology and analysis of epidemiology and clinical investigations relating to the etiology and pathogenesis of non-insulin dependent diabetes (NIDDM) and its complications. The center will assist in the development and application of standardized methods for epidemiological and clinical investigations, and data analysis relating to diabetes and collaborate with those interested in applying such techniques elsewhere. The Center will advise and help in the design of new studies, including onsite assistance when necessary.

The center serves as a central laboratory for the WHO Multicenter Study of Vascular Disease in Diabetes, as well as being a participating center for this study which is examining the mortality and incidence of vascular complications of diabetes among different ethnic groups in different countries. In addition the center has initiated a collaborative study of impaired glucose tolerance in China, and is collaborating in the preparation of a survey manual for diabetes mellitus on behalf of WHO.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69025-03 PECR

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Treatment of Impaired Glucose Tolerance in Malmohus County Sweden

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: W.C. Knowler Chief DAES, NIDDK

## COOPERATING UNITS (if any)

Lund University, Dalby, Sweden (Foreign)

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Diabetes and Arthritis Epidemiology Section

## INSTITUTE AND LOCATION

NIDDK, NIH, Phoenix, Arizona 85014

## TOTAL MAN-YEARS:

0.1

## PROFESSIONAL:

0.1

## OTHER:

0.0

## CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects☐ (b) Human tissues☒ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Mortality according to glucose tolerance was studied to determine the prognosis of impaired glucose tolerance. In 1962-65, 228, 833 subjects were screened for glycosuria. Of 2477 with glycosuria, 2180 were given oral glucose tolerance tests and grouped according to normal tolerance, impaired glucose tolerance, or diabetes by World Health Organization criteria. Among subjects at least 25 years old with normal tolerance, impaired glucose tolerance, or diabetes, age-sex-adjusted mortality through 1983 was  $39 \pm 2$ ,  $49 \pm 4$ , and  $71 \pm 4$  deaths/1000 person-years ( $\pm$  standard error) for all causes ( $p < .001$ ) for difference in 3 groups), and  $24 \pm 2$ ,  $25 \pm 3$ , and  $40 \pm 3$  for vascular causes (cardiovascular, cerebrovascular, or renal disease) ( $p < .001$ ). 206 men with abnormal tolerance by local, but not World Health Organization, criteria were randomly assigned to diet with tolbutamide, diet only, or no treatment, which was continued through 1975. Age-adjusted all-cause mortality through 1983 did not differ significantly among treatment groups ( $34 \pm 9$ ,  $52 \pm 10$ ,  $45 \pm 19$ ), but vascular mortality was  $10 \pm 5$ ,  $31 \pm 8$ , and  $38 \pm 19$  in those assigned to tolbutamide, diet only, or no treatment ( $p < .05$ ). Thus compared with persons with normal tolerance, diabetic subjects had higher all-cause and vascular mortality, and those with impaired glucose tolerance had higher all-cause but similar vascular mortality. Treatment of abnormal glucose tolerance apparently reduced vascular but not total mortality.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69026-03 PECR

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Carbohydrate and Energy Metabolism in Human Muscle

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: C. Bogardus Chief CDNS, NIDDK  
Others: B. Nyomba Visiting Associate CDNS, NIDDK  
I. Raz Special Volunteer CDNS, NIDDK

## COOPERATING UNITS (if any)

Indian Health Service; Dept. of Kinesiology, Univ. of Illinois, Urbana, IL (A. Katz)

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Clinical Diabetes and Nutrition Section

## INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

## TOTAL MAN-YEARS:

0.1

## PROFESSIONAL:

0.1

## OTHER:

0.0

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Studies were performed to investigate the regulation of glycolysis, glucose oxidation, and energy metabolism in human skeletal muscle during exercise, anoxia, and hyperinsulinemia. In particular, investigations were focused on glucose 1,6-bisphosphate (GP2) as a possible important regulator of glycolysis, since previous studies had shown it to increase in parallel with glycolysis after isometric contraction of the muscle to fatigue. Preliminary results have suggested that GP2 increases in human skeletal muscle following either isometric contraction, anoxia, circulatory occlusion, and in response to hyperinsulinemia. GP2 activation may be a result of activation of GP2 synthase by its substrates glucose 1-phosphate (G1P) and glucose 6-phosphate (G6P). Thus, it appears that GP2 may have a significant role to play in stimulating glycolysis under a variety of metabolic conditions in human skeletal muscle.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69027-02 PECR

PERIOD COVERED  
October 1, 1988 to September 30, 1989TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Role of Insulin Receptor Tyrosine Kinase in Insulin Resistance in Pima Indians

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	C. Bogardus	Chief	CDNS, NIDDK
Others:	D.M. Mott	Research Chemist	CDNS, NIDDK
	B.L. Nyomba	Visiting Associate	CDNS, NIDDK
	B. Swinburn	Visiting Associate	CDNS, NIDDK
	V. Ossowski	Biologist	CDNS, NIDDK

## COOPERATING UNITS (if any)

Indian Health Service

LAB/BRANCH  
Phoenix Epidemiology and Clinical Research BranchSECTION  
Clinical Diabetes and Nutrition SectionINSTITUTE AND LOCATION  
NIDDK/NIH, Phoenix, Arizona 85016TOTAL MAN-YEARS:  
0.8PROFESSIONAL:  
0.8OTHER:  
0.0

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Since insulin receptor tyrosine kinase may be required for insulin action, we investigated the possible role of the kinase in the pathogenesis of insulin resistance in Pima Indians.

The kinase activity was determined in muscle biopsies obtained before and during a two-step euglycemic, hyperinsulinemic clamp in 6 diabetic and 14 non-diabetic male Pima Indians. The kinase activity increased with insulin infusion in vivo in a dose-dependent manner and its stimulation by insulin in diabetics was less than in non-diabetic subjects. However, there was no direct relationship between the kinase activity and insulin action. On the contrary, the sensitivity of the kinase to insulin was inversely related to insulin action and directly related to plasma insulin levels.

In another study, we induced changes in insulin action in 6 non-diabetic Pima Indians and 6 non-diabetic Caucasians by feeding them each of a "traditional" high carbohydrate, Pima-type diet, and a "modern," high-fat Western-type diet for two weeks. We then determined the receptor kinase activity and estimated insulin action by the minimal model method. The modern diet induced insulin resistance and increased the kinase activity in the majority of subjects. The changes in kinase activity were negatively correlated with the changes in insulin action and positively correlated with plasma insulin levels.

In conclusion, the insulin receptor tyrosine kinase activity, while defective in type II diabetes, is not directly associated with insulin action in non-diabetic subjects and is not the site of in vivo insulin resistance in Pima Indians. It is possible that the kinase increases with plasma insulin levels as part of a mechanism compensating for insulin resistance at a distal site.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69028-01 PECR

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Genetics of Non-insulin Dependent Diabetes Mellitus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	W.C. Knowler	Chief,	DAES, NIDDK
Others:	P.H. Bennett	Chief	PECRB, NIDDK
	C. Bogardus	Chief	CDNS, NIDDK
	D.J. Pettitt	Assistant Chief	DAES, NIDDK
	S. Lillioja	Visiting Scientist	CDNS, NIDDK

## COOPERATING UNITS (if any)

Collaborative Research, Waltham, MA, Bowman-Gray Medical School, Winston-Salem, NC  
(D. Bowden)

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Diabetes and Arthritis Epidemiology Section

## INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85014

## TOTAL MAN-YEARS:

0.2

## PROFESSIONAL:

0.1

## OTHER:

0.1

## CHECK APPROPRIATE BOX(ES)

- |   |   |                                      |
|---|---|--------------------------------------|
| <input type="checkbox"/> (a) Human subjects | <input checked="" type="checkbox"/> (b) Human tissues | <input type="checkbox"/> (c) Neither |
| <input type="checkbox"/> (a1) Minors        |   |                                      |
| <input type="checkbox"/> (a2) Interviews    |   |                                      |

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Non-insulin dependent diabetes mellitus is a common chronic disease that develops in most populations in late middle age. The Pima Indians of Arizona have the highest reported prevalence of this disease in the world and in contrast to many populations the disease often presents at an earlier age. As a result of long-term epidemiological studies in the total population the familial nature of the disease has been well documented and segregation analyses suggest the possibility inheritance as a single co-dominant major gene. This project will search for genetic determinants of NIDDM using the techniques of genetic linkage analysis with restriction fragment length polymorphism markers (RFLPs) to identify the chromosomal location of inherited determinants of NIDDM in the Pima Indian population. A number of informative pedigrees have been identified and lymphoblast cell lines from informative members of these pedigrees are being established. DNA from these lymphoblasts will be isolated and polymorphic probes will be applied to search for evidence of linkage of these markers and NIDDM. Probes with established chromosomal locations will be used to screen the genome to detect genetic linkage with NIDDM.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69029-01 PECR

PERIOD COVERED  
October 1, 1988 to September 30, 1989TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Regulation of Skeletal Muscle Ribosomal Protein S6 Kinase by Insulin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. Sommercorn	Senior Staff Fellow	CDNS, NIDDK
Others:	C. Bogardus	Chief	CDNS, NIDDK
	R. Fields	Biologist	CDNS, NIDDK
	I. Raz	Special Volunteer	CDNS, NIDDK
	F. Zurlo	Visiting Fellow	CDNS, NIDDK

COOPERATING UNITS (if any)

Indian Health Service

LAB/BRANCH  
Phoenix Epidemiology and Clinical Research BranchSECTION  
Clinical Diabetes and Nutrition SectionINSTITUTE AND LOCATION  
NIDDK/NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS: 0.2	PROFESSIONAL: 0.2	OTHER: 0.0
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CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Ribosomal protein S6 kinase is activated by insulin in a variety of biological systems. Phosphorylation of the S6 protein is thought to contribute to the regulation of protein synthesis by enhancing formation of the initiation complex for translation. Other studies undertaken at the Clinical Diabetes and Nutrition Section have shown that lower rates of glucose utilization in skeletal muscle of subjects with insulin resistance can be explained in part by abnormal regulation of glycogen synthase and glycogen synthase phosphatase by insulin. These abnormalities are not explained by defects in the insulin receptor. Thus, the biochemical lesion in the mechanism of insulin action appears to be between the receptor and glycogen synthase phosphatase activity. The S6 kinase project was undertaken to determine if similar defects in insulin action could be observed in a metabolic pathway (protein synthesis) distinct from glucose metabolism. Adult Caucasian and Pima Indian volunteers were subjected to a hyperinsulinemic, euglycemic clamp and thigh muscle biopsies were taken before and at 15-minute intervals up to 90 minutes of insulin infusion. S6 kinase activity was determined in high-speed extracts of the muscle. In subjects with impaired glucose tolerance (IGTs) the basal S6 kinase is lower than in normal subjects and the activity is increased by insulin infusion to only 50% of the maximum activation observed in subjects with normal glucose tolerance. Furthermore, the response is delayed in the IGTs. The response in diabetic subjects is similar to that of the IGTs. It appears that the biochemical lesion in insulin action expressed in subjects with impaired glucose tolerance is common to the mechanisms by which insulin regulates glycogen synthesis and protein synthesis.

Z01

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69030-01 PEGR

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Contribution of Protein Tyrosine Phosphatase to Insulin Resistance

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. Sommercorn	Senior Staff Fellow	CDNS, NIDDK
Others:	C. Bogardus	Chief	CDNS, NIDDK
	R. Maeda	Visiting Fellow	CDNS, NIDDK
	I. Raz	Special Volunteer	CDNS, NIDDK

## COOPERATING UNITS (if any)

Indian Health Service

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Clinical Diabetes and Nutrition Section

## INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

## TOTAL MAN-YEARS:

0.1

## PROFESSIONAL:

0.1

## OTHER:

0.0

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The receptor for insulin is a protein tyrosine kinase that is stimulated by binding of insulin. Substantial evidence supports the conclusion that the enzymatic activity of the receptor is essential for transmitting the insulin signal intracellularly. Other studies undertaken at the Clinical Diabetes and Nutrition Section have shown abnormal regulation of glycogen synthase, glycogen synthase phosphatase, and S6 kinase activities in skeletal muscle of subjects with impaired glucose tolerance. These downstream abnormalities do not appear to be explained by abnormal insulin binding in skeletal muscle of these subjects. Nor does there appear to be a defect in the protein tyrosine kinase activity of the insulin receptor that could explain the differences. The present project was initiated to determine if subjects with impaired glucose tolerance have abnormally high protein tyrosine phosphatase activity in skeletal muscle that would attenuate the signal generated by the insulin receptor. Work to date has focused on characterizing the human skeletal muscle protein tyrosine phosphatases in terms of stability to dilution and influence the EDTA on activity. The results are consistent with those reported for the protein tyrosine phosphatase activities isolated from human placenta.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69031-01 PECR

PERIOD COVERED  
October 1, 1988 to September 30, 1989TITLE OF PROJECT (90 characters or less. Title must fit on one line between the borders.)  
Regulation of Phosphorylase Phosphatase by Insulin

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI: J. Sommercorn	Senior Staff Fellow	CDNS, NIDDK
Others: C. Bogardus	Chief	CDNS, NIDDK
D. Mott	Research Chemist	CDNS, NIDDK
R. Maeda	Visiting Fellow	CDNS, NIDDK
I. Raz	Special Volunteer	CDNS, NIDDK

COOPERATING UNITS (if any)  
Indian Health ServiceLAB/BRANCH  
Phoenix Epidemiology and Clinical Research BranchSECTION  
Clinical Diabetes and Nutrition SectionINSTITUTE AND LOCATION  
NIDDK/NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS: 0.1	PROFESSIONAL: 0.1	OTHER: 0.0
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## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In cultured fibroblasts, insulin is known to rapidly increase the activity of phosphoprotein phosphatase activity measured with phosphorylase a as a substrate. The activity was identified as a type I phosphatase by its sensitivity to inhibitor 2. Other work at the Clinical Diabetes and Nutrition Section has shown a similar insulin stimulation of phosphatase activity in human skeletal muscle. The phosphatase was assayed with glycogen synthase as a substrate. The timecourse and magnitude of activation were similar to the results obtained in fibroblasts. The present study was undertaken to determine if the stimulated phosphatase in human muscle could be assayed with phosphorylase a as a substrate and to determine the subcellular distribution of the activated phosphatase and its sensitivity to inhibitor 2. Subjects are adult Pima Indians and lean Caucasians who have been subjected to a hyperinsulinemic, euglycemic clamp with thigh muscle biopsies taken at 0, 15, 30, 45, 60, and 90 minutes of insulin infusion. After lyophilization, the muscle is homogenized and post-mitochondrial supernatant, cytosol, and glycogen fractions are prepared by centrifugation. Phosphorylase phosphatase is assayed in all three fractions using 32P-labeled phosphorylase a as a substrate. In subjects with normal glucose tolerance insulin infusion stimulates phosphorylase phosphatase activity in all fractions about 50-100%. The maximum effect is observed at 45 minutes after commencing insulin infusion. This timecourse is delayed relative to the response of glycogen synthase phosphatase in similar subjects. Thus, there may be more than one serine/threonine protein phosphate activated by insulin in human skeletal muscle. Alternatively, one phosphatase may be activated but its substrate specificity may change with time after activation. We have not yet determined if the insulin-activated phosphorylase phosphatase is a type I phosphatase or if the response is abnormal in subjects with impaired glucose tolerance or NIDDM.

## DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69032-01 PECR

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (20 characters or less. Title must fit on one line between the borders.)

Regulation of Skeletal Muscle Casein Kinase II by Insulin

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J. Sommercorn	Senior Staff Fellow	CDNS, NIDDK
Others:	C. Bogardus	Chief	CDNS, NIDDK
	R. Fields	Biologist	CDNS, NIDDK
	R. Maeda	Visiting Fellow	CDNS, NIDDK
	I. Raz	Special Volunteer	CDNS, NIDDK
	F. Zurlo	Visiting Fellow	CDNS, NIDDK

## COOPERATING UNITS (if any)

Indian Health Service

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Clinical Diabetes and Nutrition Section

## INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

## TOTAL MAN-YEARS:

0.9

## PROFESSIONAL:

0.9

## OTHER:

0.0

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects      ☐ (b) Human tissues      ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unexpanded type. Do not exceed the space provided.)

Casein kinase II (CKII) is a serine/threonine protein kinase that is activated in response to insulin in several cell culture lines. The contribution of CKII to the mechanism of insulin action is not known, but there is reason to believe that it could contribute to coordinating a variety of metabolic effects of insulin. The case for this belief is particularly strong for the regulation of gene expression. Glycogen synthase is a physiological substrate of CKII and thus the kinase may contribute directly to its regulation. CKII may also contribute indirectly to regulation of glycogen synthase by influencing the activity of a glycogen synthase phosphatase activity which activates glycogen synthase. Other studies undertaken at the Clinical Diabetes and Nutrition Section have shown that the responses of glycogen synthase and glycogen synthase phosphatase are abnormal in skeletal muscle of subjects with impaired glucose tolerance. Because CKII may contribute to the activation of glycogen synthase phosphatase, we initiated this project to determine if CKII is activated in skeletal muscle of normal subjects, and if the response is abnormal in subjects with impaired glucose tolerance. Adult Caucasian and Pima Indian volunteers were subjected to a hyperinsulinemic, euglycemic clamp and thigh muscle biopsies were taken before and at 15-minute intervals up to 90 minutes of insulin infusion. CKII activity was determined in high-speed extracts of the muscle. Results to date demonstrate that CKII is activated about 50-100% in normal subjects after 30 minutes of insulin infusion. Activity then declines to basal levels by 90 minutes. We do not have data yet on subjects with impaired glucose tolerance or NIDDM.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69033-01 PECR

## PERIOD COVERED

October 1, 1988 to September 30, 1989

## TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Relationship Between Insulin Resistance &amp; Blood Pressure in Different Ethnic Groups

## PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: C. Bogardus Chief CDNS, NIDDK

Others: M. F. Saad Visiting Associate DAES, NIDDK

## COOPERATING UNITS (if any)

Indian Health Service; Diabetes and Arthritis Epidemiology Section (DAES)

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Clinical Diabetes and Nutrition Section

## INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

## TOTAL MAN-YEARS:

0.1

## PROFESSIONAL:

0.1

## OTHER:

0.0

## CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither  
☐ (a1) Minors  
☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

It has been recently proposed that insulin is the link between obesity, hypertension, and glucose intolerance. Both obesity and glucose intolerance are associated with increased insulin resistance, hyperinsulinemia, and increased prevalence of hypertension. In addition, patients with essential hypertension are insulin-resistant and hyperinsulinemic. Therefore, it has been postulated that insulin might play a role in the pathogenesis of hypertension through stimulation of the sympathetic nervous system, promoting renal sodium retention, or affecting cation transport. However, in a cross-sectional study of 3024 Pima Indians seen at the NIH research clinic, there was no relationship between hypertension or blood pressure and serum insulin concentrations. To explore this issue further, the relationship between both insulin resistance and insulin concentrations and blood pressure is being studied in Pima Indians compared to other ethnic groups (Caucasians and Blacks).

## DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

## NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DK 69034-01 PECR

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
The Long Q-T Interval Syndrome in Diabetes Mellitus

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: C. Bogardus

Chief

CDNS, NIDDK

Others: M.F. Saad

Visiting Associate

DAES, NIDDK

## COOPERATING UNITS (if any)

Indian Health Service; Diabetes and Arthritis Epidemiology Section (DAES)

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Clinical Diabetes and Nutrition Section

## INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

TOTAL MAN-YEARS:

0.1

PROFESSIONAL:

0.1

OTHER:

0.0

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects☐ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Recent studies indicated that diabetic autonomic neuropathy can lead to the long Q-T interval syndrome. This may increase the susceptibility of patients with diabetic autonomic neuropathy to ventricular arrhythmias and subsequent sudden death. The prevalence of and the risk factors for this condition will be determined in diabetic and non-diabetic Pima Indians by reviewing the electrocardiograms and the records of 1,000 adults seen at the NIH research clinic. In addition, 50 diabetic subjects with long Q-T interval, 50 diabetic subjects with normal Q-T interval, and 50 non-diabetic subjects with normal electrocardiograms will be studied in detail. All subjects will undergo detailed evaluation of the autonomic nervous system using a battery of standardized tests. Ambulatory cardiac monitoring will be obtained to determine the frequency of silent ischemic episodes and ventricular premature beats.

## NOTICE OF INTRAMURAL RESEARCH PROJECT

Z01 DK 69035-01 PECR

## PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)  
Autonomic Nervous System Activity in Obesity

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: C. Bogardus

Chief

CDNS, NIDDK

Others: M.F. Saad

Visiting Associate

DAES, NIDDK

## COOPERATING UNITS (if any)

Indian Health Service; Diabetes and Arthritis Epidemiology Section (DAES)

## LAB/BRANCH

Phoenix Epidemiology and Clinical Research Branch

## SECTION

Clinical Diabetes and Nutrition Section

## INSTITUTE AND LOCATION

NIDDK/NIH, Phoenix, Arizona 85016

## TOTAL MAN-YEARS:

0.1

## PROFESSIONAL:

0.1

## OTHER:

0.0

## CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects☐ (b) Human tissues☐ (c) Neither☐ (a1) Minors☐ (a2) Interviews

## SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The Pima Indians have a high prevalence of obesity with more than 70% of the adult population having a body mass index  $27 \text{ kg/m}^2$ . Alteration in autonomic nervous system activity may contribute to the pathogenesis of obesity in this population. Studies in animal models of spontaneous obesity demonstrated decreased sympathetic nervous system activity. A recent study in obese humans showed decreased sympathetic and parasympathetic activity. The authors postulated that a disordered homeostatic mechanism promotes an excessive storage of energy by decreasing sympathetic activity. The relationship between obesity and autonomic nervous system activity will be studied in 50 Pima Indians aged 20-40 years. Fifty Caucasians of comparable age and weight will be included for comparison.

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